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(54) Title: CONTROLLED DISSOLUTION CROSS–LINKED PROTEIN CRYSTALS

(57) Abstract

The present invention relates to cross–linked protein crystals characterized by the ability to change from insoluble and stable form to soluble and active form upon a change in the environment of said crystals, said change being selected from the group consisting of change in temperature, change in pH, change in chemical composition, change from concentrate to dilute form, change in oxidation–reduction potential of the solution, change in the incident radiation, change in transition metal concentration, change in fluoride concentration, change in free radical concentration, change in metal chelater concentration, change in shear force acting upon the crystals and combinations thereof. According to one embodiment of this invention, such cross–linked protein crystals are capable of releasing their protein activity at a controlled rate. This invention also provides methods for producing such cross–linked protein crystals, methods using them for protein delivery and methods using them in cleaning agents, including detergents, pharmaceutical compositions, vaccines, personal care compositions, including cosmetics, veterinary compositions, foods, feeds, diagnostics and formulations for decontamination.

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CONTROLLED DISSOLUTION CROSSLINKED PROTEIN CRYSTALS

TECHNICAL FIELD OF THE INVENTION

The present invention relates to crosslinked protein crystals characterized by the ability to change from insoluble and stable form to soluble and active form upon a change in the environment surrounding said crystals, said change being selected from the group consisting of change in temperature, change in pH, change in chemical composition, change from concentrate to dilute form, change in oxidation-reduction potential of the solution, change in the incident radiation, change in transition metal concentration, change in flouride concentration, change in free radical concentration, change in metal chelater concentration, change in shear force acting upon the crystals and combinations thereof. According to one embodiment of this invention, such crosslinked protein crystals are capable of releasing their protein activity at a controlled rate. This invention also provides methods for producing such crosslinked protein crystals, methods using them for protein delivery, methods for using them in cleaning agents, including detergents, therapeutic formulations, pharmaceutical compositions, vaccines, personal care compositions, including

cosmetics, veterinary compositions, foods, feeds, diagnostics and formulations for decontamination.

BACKGROUND OF THE INVENTION

Proteins are used in a wide range of applications in the fields of industrial chemistry, pharmaceuticals, veterinary products, cosmetics and other consumer products, foods, feeds, diagnostics and decontamination. At times, such uses have been limited by constraints inherent in proteins themselves or imposed by the environment or media in which they are used. Such constraints may result in poor stability of the proteins, variability of performance or high cost. In order for proteins to realize their full potential in the fields in which they are used, they must be able to function without excessive intervention by their surrounding environment. In the past, environmental elements have often posed barriers to the widespread use of proteins.

Various approaches have been employed to overcome these barriers. However, these approaches have incurred either loss of protein activity or the additional expense of protein stabilizing carriers or formulations.

One unique approach to overcoming barriers to the widespread use of proteins is crosslinked enzyme crystal ("CLEC™) technology [N.L. St. Clair and M.A. Navia, J. Am. Chem. Soc., 114, pp. 4314-16 (1992)]. Crosslinked enzyme crystals retain their activity in environments that are normally incompatible with enzyme function. Such environments include prolonged exposure to proteases and other protein digestion agents, high

temperature or extreme pH. In such environments, crosslinked enzyme crystals remain insoluble and stable.

Protein solubility, leading to controlled release or dissolution of protein, is important in many industrial and medical fields. Such fields include those concerning cleaning agents, including detergents, pharmaceuticals, including therapeutics and vaccines, consumer and personal care products, veterinary products, foods, feeds, diagnostics and decontamination. Various approaches to controlled release have been proposed. These include encapsulation, such as that described in United States patents 4,579,779 and 5,500,223. Other approaches include the use of mechanical or electrical feed devices and osmotic pumps.

Controlled release in the pharmaceutical field has been addressed by various means. United States patent 5,569,467 refers to the use of sustained release microparticles comprising a biocompatible polymer and a pharmaceutical agent, which is released as the polymer degrades. United States patent 5,603,956 refers to solid, slow release pharmaceutical dosage units comprising crosslinked amylase, alpha amylase and a pharmaceutical agent. United States patent 4,606,909 refers to oral, controlled-release multiple unit formulations in which homogeneous cores containing particles of sparingly soluble active ingredients are coated with a pH-sensitive erodable coating. United States patent 5,593,697 refers to pharmaceutical or veterinary implants comprising a biologically active material, an excipient comprising at least one water soluble material and at least one water insoluble material and a polymer film coating

adapted to rupture at a predetermined period of time after implant.

The objective of controlled release of proteins, however, must be balanced with the fact that the protein itself may not be stable under storage conditions. Protein stability may also be adversely affected by other components of the formulation in which it is contained. For example, heavy duty liquid detergents constitute hostile environments for component enzymes. Such problems have been approached through the use of mutant subtilisin proteases, which are said to have improved oxidative stability. See United States patent 4,760,025 and PCT patent application WO89/06279. Proteins, the enzymes most widely used in detergents, catalyze their own decomposition. Strategies such as the addition of protease inhibitors (e.g., borate with glycols) or the lowering of water activity have been only partially effective.

Another approach, described in United States patent 5,385,959, is encapsulation of degradation-sensitive detergent components in capsules of composite emulsion polymers, which permit dilution release thereof. United States patent 5,286,404 refers to a liquid detergent composition said to have improved enzyme solubility while preserving enzyme activity. The improvement is attributed to chemical modification of free primary amino groups in an enzyme solution via aldehyde treatment, acylation or alkylation.

Despite such progress in protein technology generally, the need still exists for proteins which are stable under conditions of storage, while active under conditions of use.

DISCLOSURE OF THE INVENTION

The present invention relates to crosslinked protein crystals characterized by the ability to change from an insoluble and stable form to a soluble and active form upon a change in the environment surrounding said crystals, said change being selected from the group consisting of change in temperature, change in pH, change in chemical composition, change from concentrate to dilute form, change in oxidation-reduction potential of the solution, change in the incident radiation, change in transition metal concentration, change in flouride concentration, change in free radical concentration, change in metal chelater concentration, change in shear force acting upon the crystals and combinations thereof. According to one embodiment of this invention, such crosslinked protein crystals are capable of releasing their protein activity at a controlled rate.

Advantageously, crosslinked protein crystals according to this invention are insoluble and stable under storage conditions and soluble and active under conditions of use.

This invention also provides cleaning agents, including detergents, therapeutic proteins pharmaceutical compositions, vaccines, personal care compositions, including cosmetics, veterinary compositions, foods, feeds, diagnostics and formulations for decontamination. Additionally, this invention includes methods for producing such crosslinked protein crystals and methods for protein delivery using them.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph representing the stability of various enzymes in Ciba detergent # 16 at 40°C.

Figure 2 is a graph representing the washing performance of liquid detergent formulations, including a formulation containing crosslinked subtilisin crystals according to the present invention, on fabric soiled with blood, milk and carbon black.

Figure 3 is a graph representing the washing performance of liquid detergent formulations, including a formulation containing crosslinked subtilisin crystals according to the present invention, after storage at 30°C, on fabric soiled with cocoa.

Figure 4 is a graph representing the washing performance of liquid detergent formulations, including a formulation containing crosslinked subtilisin crystals according to the present invention, after storage at 40°C, on fabric soiled with cocoa.

Figure 5 is a graph representing the washing performance of liquid detergent formulations, including a formulation containing crosslinked subtilisin crystals according to the present invention, after storage at 30°C, on fabric soiled with blood, milk and carbon black.

Figure 6 is a graph representing the washing performance of liquid detergent formulations, including a formulation containing crosslinked subtilisin crystals according to the present invention, after storage at 40°C, on fabric soiled with blood, milk and carbon black.

Figure 7 is a graph representing the washing performance of liquid detergent formulations, including

a formulation containing crosslinked subtilisin crystals according to the present invention, after storage at 30°C, on fabric soiled with blood.

Figure 8 is a graph representing the solubility of crosslinked subtilisin crystals according to the present invention at 30°C.

Figure 9 is a graph representing the solubility of crosslinked subtilisin crystals according to the present invention at 37°C.

DETAILED DESCRIPTION OF THE INVENTION

In order that the invention herein described may be more fully understood, the following detailed description is set forth. In the description, the following terms or phrases are employed:

Aqueous-organic solvent mixture -- a mixture comprising n% organic solvent, where n is between 1 and 99 and m% aqueous, where m is 100-n.

Biphasic substrate -- A solution of a substrate with two distinct phases, either liquid/solid or liquid/liquid phases, one of which contains a substrate for a reaction catalyzed by the protein constituent of a crosslinked protein crystal. An emulsion of olive oil in an aqueous solution containing discrete aqueous and organic phases exemplifies a substrate for crosslinked crystals of lipase.

Catalytically effective amount -- an amount of crosslinked protein crystals of this invention which is effective to treat, protect, repair or detoxify the area to which they are applied over some period of time.

Change in chemical composition -- any change in the chemical components of the environment

surrounding the crosslinked protein crystals that affects the environment or the crosslinker, including addition of chemical reagents, chemical changes induced by application of energy in the form of light, microwave, or radiation to the environment, chemical events that affect the crosslinker and combinations thereof.

Change in shear force acting upon the crystals -- any change in factors of the environment surrounding the crosslinked protein crystals under conditions of use, such as, changes in mechanical pressure, both positive and negative, revolution stirring, centrifugation, tumbling, mechanical agitation and filtration pumping.

Controlled dissolution -- dissolution of crosslinked protein crystals or release of the protein constituent from the crystalline state to the soluble state that is (1) triggered by a change in the environment surrounding said crystals, said change being selected from the group consisting of change in temperature, change in pH, change in chemical composition, change from concentrate to dilute form, change in oxidation-reduction potential of the solution, change in the incident radiation, change in transition metal concentration, change in flouride concentration, change in free radical concentration, change in metal chelater concentration, change in shear force acting upon the crystals and combinations thereof and (2) controlled by a factor selected from the group consisting of the following: degree of crosslinking of said crosslinked protein crystals, the amino acids residues involved in the crosslinks, whether the crosslinker is homobifunctional or heterobifunctional, the length of time of exposure of protein crystals to

the crosslinking agent, the rate of addition of crosslinking agent to said protein crystals, the nature of the crosslinker, the chain length of the crosslinker, the surface area of said crosslinked protein crystals, the size of said crosslinked protein crystals, the shape of said crosslinked protein crystals and combinations thereof. As used herein, the phrase "controlled dissolution" does not include leaching.

Controlled dissolution crosslinked protein crystals -- crosslinked protein crystals that slowly dissolve after being exposed a given trigger and release the soluble form of the protein into solution. The activity of controlled dissolution crosslinked protein crystals arises primarily from the soluble form of the protein released from the crystal.

Crosslinked crystal form of protein -- crosslinked protein crystals that remain insoluble and in the solid state when added to solution.

Enhanced protein activity -- activity of the crosslinked crystal form of a protein which is enhanced as compared with the soluble form of the protein. According to various embodiments of the present invention, enhanced protein activity is exhibited by any one of the following: a crosslinked crystal form of a protein having activity that is 200-300 times higher than that of the soluble form of the protein; a crosslinked crystal form of a protein having activity that is 100-200 times higher than that of the soluble form of the protein; a crosslinked crystal form of a protein having activity that is 10-100 times higher than that of the soluble form of the protein; a crosslinked crystal form of a protein having activity that is 20-50 times higher than that of the soluble

form of the protein; a crosslinked crystal form of a protein having activity that is 10-20 times higher than that of the soluble form of the protein; a crosslinked crystal form of a protein having activity that is 20-30 times higher than that of the soluble form of the protein; a crosslinked crystal form of a protein having activity that is 5-10 times higher than that of the soluble form of the protein; a crosslinked crystal form of a protein having activity that is 2-3 times higher than that of the soluble form of the protein; a crosslinked crystal form of a protein having activity that is at least 3 times higher than that of the soluble form of the protein; a crosslinked crystal form of a protein having activity that is at least 2 times higher than that of the soluble form of the protein; a crosslinked crystal form of a protein having activity that is at least 25-99% higher than that of the soluble form of the protein; a crosslinked crystal form of a protein having activity that is at least 25-30% higher than that of the soluble form of the protein; or a crosslinked crystal form of a protein having activity that is at least 20% higher than that of the soluble form of the protein.

Formulations for decontamination -- formulations selected from the group consisting of: formulations for decontamination of chemical wastes, herbicides, insecticides, pesticides, environmental hazards and chemical warfare agents.

Insoluble and stable form of a protein -- a form of a protein which is insoluble in aqueous solvents, organic solvents or aqueous-organic solvent mixtures and which displays greater stability than the soluble form of the protein. According to an alternate embodiment of this invention, the phrase "insoluble and

stable form of a protein" may be a protein which is insoluble in dry formulations but soluble in wet formulations. In any embodiment, the crosslinked protein crystals may be active in insoluble form. And in one embodiment, the crosslinked protein crystals may be active in insoluble form, then dissolve or are removed or digested once their function is complete.

Macromolecular substrate -- a large biomolecule, such as a protein or a carbohydrate having a molecular weight of at least 600-700 Daltons, which is also a substrate for a reaction catalyzed by the protein constituent of a crosslinked protein crystal.

Organic solvents -- any solvent of non-aqueous origin.

Pharmaceutically effective amount -- an amount of crosslinked protein crystals which is effective to treat a condition in an individual to whom they are administered over some period of time.

Prophylactically effective amount -- an amount of crosslinked protein crystals which is effective to prevent a condition in an individual to whom they are administered over some period of time.

Protein -- a protein or, alternatively, a glycoprotein or, alternatively, any peptide having a tertiary structure.

The protein constituents of the crosslinked protein crystal formulations of this invention may be naturally or synthetically modified. They may be glycoproteins, phosphoproteins, sulphoproteins, iodoproteins, methylated proteins, unmodified proteins or contain other modifications.

The protein constituent of the crosslinked protein crystal formulations of this invention may be any protein including, for example, hormones, such as

parathyroid hormone, enzymes, antibodies, viral receptors, viral surface glycoproteins, parasite glycoproteins, parasite receptors, T-cell receptors, MHC molecules, immune modifiers, tumor antigens, mucins, inhibitors, growth factors, trophic factors, cytokines, lymphokines, cytokines, toxoids, nerve growth hormones, blood clotting factors, adhesion molecules, multidrug resistance proteins, adenylate cyclases, bone morphogenic proteins and lectins.

Also included among proteins are glycoprotein hormones and cytokines. Examples of hormones include follicle stimulating hormone, human chorionic gonadotropin, luteinizing hormone, thyrotrophin and ovine, bovine, porcine, murine and rat alleles of these hormones. Examples of cytokine glycoproteins include α-interferon, lymphotoxin, and interleukin-2. Also included are glycoprotein tumor-associated antigens, for example, carcinoembryonic antigen (CEA), human mucins, her-2/neu, and prostate-specific antigen (PSA) [R.A. Henderson and O.J. Finn, Advances in Immunology, 62, pp. 217-56 (1996)].

Protein activity -- an activity selected from the group consisting of binding, catalysis, or activities which generate a functional response within the environment in which the protein is used, such as the induction of an immune response and immunogenicity or hydrolysis of lipids in lipase deficient individuals, or combinations thereof.

Protein activity release rate -- the quantity of protein dissolved per unit time.

Soluble form of protein -- individual protein molecules in solution and dissociated from a crystal lattice.

Small molecule substrate -- molecules having molecular weights generally less than 600 Daltons which are also substrates for reactions catalyzed by the protein constituents of crosslinked protein crystals.

Therapeutic protein -- A protein which is administered to a patient in a pharmaceutical formulation and manner. Therapeutic proteins include, for example, hormones, enzymes including lipase, antibodies, viral receptors, T-cell receptors, chemokines, chemokine receptors, MHC molecules, tumor antigens, mucins, inhibitors, growth factors, trophic factors, cytokines, lymphokines, toxoids, nerve growth hormones, blood clotting factors, adhesion molecules, multidrug resistance proteins, adenylate cyclases and bone morphogenic proteins.

Vaccine antigen -- a protein derived from an infectious agent such as a virus, parasite, or tumor antigen. The protein activity of such vaccine antigens is to induce protective immunity against the infectious agent.

The crosslinked protein crystals of this invention are particularly advantageous because they are stable in harsh environments imposed by the formulations or compositions in which they are employed or conditions of their storage. At the same time, these crosslinked protein crystals are capable of controlled dissolution or release of their activity when exposed to one or more triggers in their environment. Such triggers may be selected from the group consisting of change in temperature, change in pH, change in chemical composition, change from concentrate to dilute form, change in shear force acting upon the crystals and combinations thereof.

Controlled dissolution or release of activity of crosslinked protein crystals according to this invention may also be triggered over a change in time.

Specific examples of such triggers include an increase or decrease in temperature, for example, an increase in temperature from a low temperature between about 0°C and about 20°C to a high temperature between about 25°C and about 70°C. Other triggers include a change from acidic pH to basic pH and a change from basic pH to acidic pH. Examples of triggers of change from concentrate to dilute form include, for example, a change in solute concentration, a change in concentration of all solutes from about 2-fold to about 10,000-fold, a change in concentration of all solutes from about 2-fold to about 700-fold, an increase or decrease in salt concentration, an increase or decrease in water concentration, an increase or decrease in organic solvent concentration, a decrease in protein concentration and a decrease in detergent concentration.

Additional triggers involve changes in chemical composition of the environment surrounding the crosslinked protein crystals that affect the environment or the crosslinker itself. Such changes include, for example, addition of chemical reagents, increase or decrease in organic solvent concentration, chemical events that affect the crosslinker, chemical changes induced by application of energy, including light, microwave or radiation. As explained above, any of these triggers may act in combination or in sequence with one or more of the other triggers.

Controlled dissolution of crosslinked protein crystals according to the present invention may also be effected by a change in time sufficient to permit a

protein activity release rate between about 0.1% per day and about 100% per day, a change in time sufficient to permit a protein activity release rate between about 0.01% per hour and about 100% per hour and a change in time sufficient to permit a protein activity release rate between about 1% per minute and about 50% per minute.

Crosslinked protein crystals according to this invention, therefore, include those capable of releasing their protein activity at a controlled rate upon exposure to a change in their environment, said change being selected from the group consisting of change in pH, change in solute concentration, change in temperature, change in chemical composition, change in shear force acting upon the crystals and combinations thereof. Said controlled rate of releasing protein activity may be determined by a factor selected from the group consisting of the following: degree of crosslinking of the crosslinked protein crystals, length of time of exposure of protein crystals to the crosslinking agent, the rate of addition of crosslinking agent to the protein crystals, the nature of the crosslinker, the chain length of the crosslinker, the amino acids residues involved in the crosslinks, whether the crosslinker is homobifunctional or heterobifunctional, the surface area of the crosslinked protein crystals, the size of the crosslinked protein crystals, the shape of the crosslinked protein crystals and combinations thereof.

As a result of their crystalline nature, the crosslinked protein crystals of this invention achieve uniformity across the entire crosslinked crystal volume. This uniformity is maintained by the intermolecular contacts and chemical crosslinks between

the protein molecules constituting the crystal lattice. The protein molecules maintain a uniform distance from each other, forming well-defined stable pores within the crosslinked protein crystals that facilitate access of substrate to the protein, as well as removal of product. In these crosslinked protein crystals, the lattice interactions, when fixed by chemical crosslinks, are particularly important in providing stability and preventing denaturation, especially in storage, under conditions including harsh environments created by components of compositions in which the crystals are used. At the same time, the protein crystals are crosslinked in such a way that they dissolve or release their protein activity upon exposure to a trigger in their environment encountered under conditions of use. Thus, they may be substantially insoluble and stable in a composition under storage conditions and substantially soluble and active under conditions of use of said composition.

Factors contributing to the release rate of protein activity of crosslinked protein crystals according to this invention include the degree of crosslinking of the crosslinked protein crystals, the length of time of exposure of protein crystals to the crosslinking agent, the rate of addition of crosslinking agent to the protein crystals, the length of time of exposure of protein crystals to the crosslinking agent, the nature of the crosslinker, the amino acids residues involved in the crosslinks, whether the crosslinker is homobifunctional or heterobifunctional, the chain length of the crosslinker, the surface area of the crosslinked protein crystals, the size of the crosslinked protein

crystals, the shape of the crosslinked protein crystals and combinations thereof.

In addition to their activity, crosslinked protein crystals according to this invention are particularly stable and insoluble under storage conditions, including the attendant storage temperature, storage pH, storage time, storage concentrate form, storage involving little or no shear force acting upon the crystals, or combinations thereof. Advantageously, these crosslinked protein crystals are soluble and active under conditions of use, including conditions involving change in temperature, change in pH, change in chemical composition, change from concentrate to dilute form, change in shear force acting upon the crystals and combinations thereof. Such properties make the crosslinked protein crystals of this invention particularly useful for delivery of cleaning agents, including detergents, therapeutic proteins, pharmaceuticals, personal care agents or compositions, including cosmetics, vaccines, veterinary compositions, foods, feeds, diagnostics and formulations for decontamination.

According to one embodiment, the crosslinked protein crystals of this invention are characterized by a half-life of activity under storage conditions which is greater than at least 2 times that of the soluble form of the protein that is crystallized to form the crystals that are crosslinked and activity similar to that of the soluble form of the protein under conditions of use.

According to one embodiment of this invention, crosslinked protein crystals are characterized by activity which is similar to that of

their soluble or uncrosslinked crystallized counterparts under conditions of use. Advantageously however, the crosslinked protein crystals of this invention display improved stability under storage conditions, as compared to their soluble or uncrosslinked crystallized counterpart proteins.

One advantage of controlled dissolution crosslinked protein crystals is that a trigger is required to release the soluble form of the protein from the crystal lattice. Therefore, controlled dissolution crosslinked protein crystals can be prepared and function as crosslinked protein crystals with protein activity in the solid crystalline state until a trigger is encountered. After the trigger is encountered, soluble protein is released and protein activity derives from both the crosslinked crystal form, as well as the soluble form of the protein.

According to one embodiment of this invention, advantageous properties are obtained from crosslinked protein crystals that are prepared as controlled dissolution crosslinked protein crystals, but which are not subsequently exposed to a trigger. In particular, in the absence of an appropriate trigger to initiate dissolution, such crosslinked protein crystals may exhibit enhanced protein activity to macromolecular substrates, biphasic substrates or small molecule substrates as compared with their soluble counterparts. For example, lipase crystals crosslinked with sulfosuccinimidyl-6-[α-methyl-α-(2-pyridyldithio) toluamido]hexanoate (Sulfo-LC-SMPT), which are not subsequently exposed to a trigger to break the -S-S- bonds of the crosslinks, exhibit enhanced hydrolysis activity toward a biphasic olive oil substrate.

More particularly, lipase crystals crosslinked with sulfo-LC-SMPT, are characterized by about a five to ten fold higher protein activity for hydrolysis of an olive oil sustrate than the soluble form of the protein that is crystallized to form the crystals that are crosslinked. In addition, lipase crystals crosslinked with 1-ethyl-3-[3-dimethylaminoproplyl]carbodiimide hydrochloride (EDC) are characterized by about almost two fold higher protein activity for hydrolysis of an olive oil sustrate than the soluble form of the protein that is crystallized to form the crystals that are crosslinked.

The protein constituent of the crosslinked protein crystals of this invention may be any protein, including, for example, therapeutic proteins, prophylactic proteins, including antibodies, cleaning agent proteins, including detergent proteins, personal care proteins, including cosmetic proteins, veterinary proteins, food proteins, feed proteins, diagnostic proteins and decontamination proteins. Included among such proteins are enzymes, such as, for example, hydrolases, isomerases, lyases, ligases, transferases and oxidoreductases. Examples of hydrolases include thermolysin, elastase, esterase, lipase, nitrilase, amylase, pectinase, subtilinase, hydantoinase, asparaginase, urease, subtilisin and other proteases and lysozyme. Examples of lyases include aldolases and hydroxynitrile lyase. Examples of oxidoreductases include peroxidase, laccase, glucose oxidase, alcohol dehydrogenase and other dehydrogenases. Other enzymes which may be crystallized and crosslinked include cellulases and oxidases.

Examples of therapeutic or prophylactic proteins include hormones, enzymes, including lipase,

antibodies, inhibitors, growth factors, trophic factors, cytokines, lymphokines, toxoids, erythropoietin, Factor VIII, insulin, glucagon like peptide-I (insulinotropin), amylin, TPA, dornase-α, α-1-antitripsin, human growth hormones, nerve growth hormones, parathyroid hormone, bone morphogenic proteins, urease, toxoids, fertility hormones, FSH, LSH, postridical hormones, tetanus toxoid, diptheria toxoid.

The crosslinked protein crystals of this invention may be used in any of a number of chemical processes. Such processes include industrial and research-scale processes, such as organic synthesis of specialty chemicals and pharmaceuticals. Enzymatic conversion processes include oxidations, reductions, additions, including esterifications and transesterifications, hydrolyses, eliminations, rearrangements, and asymmetric conversions, including stereoselective, stereospecific and regioselective reactions.

Thus, crosslinked protein crystals according to this invention may be advantageously used instead of conventional soluble or immobilized proteins in cleaning agents, including detergents, pharmaceuticals, therapeutics, veterinary compounds, personal care compositions, including cosmetics, foods, feeds, vaccines, pulp, paper and textile processing, diagnostics and formulations for decontamination.

Crosslinked protein crystals according to this invention may also be used in various environmental applications. They may be used in place of conventional soluble or immobilized proteins for environmental purposes, such wide area decontamination of environmental hazards.

Alternatively, the crosslinked protein crystals of this invention may be used in cleaning agents, selected from the group consisting of detergents, such as powdered detergents and liquid detergents, bleaches, household cleaners, hard surface cleaners, industrial cleaners and carpet and upholstery shampoos.

Cleaning agents containing crosslinked protein crystals according to the present invention may also comprise compounds conventionally included in such agents. See, for example, Soaps and Detergents, A Theoretical and Practical Review, Louis Spitz (Ed.), AOCS Press (Champlain, Illinois) (1996). Such compounds include anionic, non-ionic, cationic or zwitterionic surfactants, or mixtures thereof.

Anionic surfactants are exemplified by alkyl sulfates, alkyl ether sulfates, alkyl sulfonates, alkylaryl sulfonates, olefin sulfonates, alkyl ether phosphates, alkyl ether phosphates, fatty acid salts, soaps, isothionates and sulfonated unsaturated esters and acids.

Non-ionic surfactants are exemplified by products of condensation of an organic aliphatic or alkyl aromatic hydrophobic compound with an alkylene oxide, alkyl polyglucosides and sugar esters.

Cationic surfactants are exemplified by quarternary ammonium salts of tertiary alkyl amines, amino amides, amino esters or imidazolines containing al least one long chain (C_8-C_{22}) aliphatic group or an alkyl-aryl group, wherein alkyl comprises about 4 to 12 carbon atoms and aryl is preferably a phenylene group.

Zwitterionic surfactants are exemplified by derivatives of quarternary ammonium, quarternary phosphonium or tertiary sulfonium compounds,

derivatives of secondary and tertiary amines and derivatives of heterocyclic secondary and tertiary amines.

And crosslinked protein crystals according to this invention may be used as ingredients in personal care compositions, including cosmetics, such as creams, lotions, emulsions, foams, washes, compacts, gels, mousses, slurries, powders, sprays, pastes, ointments, salves, balms, drops, shampoos, and sunscreens. In topical creams and lotions, for example, they may be used as humectants or for skin protection, softening, bleaching, cleaning, deproteinization, lipid removal, moisturizing, decoloration, coloration or detoxification. They may also be used as anti-oxidants in cosmetics.

According to this invention, any individual, including humans and other mammals, may be treated in a pharmaceutically acceptable manner with a pharmaceutically effective or a catalytically effective amount of crosslinked protein crystals for a period of time sufficient to treat a condition in the individual to whom they are administered over some period of time. Alternatively, individuals may receive a prophylactically effective or a catalytically effective amount of crosslinked protein crystals of this invention which is effective to prevent a condition in the individual to whom they are administered over some period of time.

Such crosslinked protein crystals may be administered alone, as part of a pharmaceutical, personal care or veterinary preparation or as part of a prophylactic preparation, such as a vaccine, with or without adjuvant. They may be administered by parenteral or non-parenteral route. For example, they

may be administered by oral, pulmonary, nasal, aural, anal, dermal, ocular, intravenous, intramuscular, intraarterial, intraperitoneal, mucosal, sublingual, subcutaneous, or intracranial route. In either pharmaceutical, personal care or veterinary applications, crosslinked protein crystals may be topically administered to any epithelial surface. Such epithelial surfaces include oral, ocular, aural, anal and nasal surfaces, to treat, protect, repair or detoxify the area to which they are applied.

The present invention also includes controlled release formulations comprising crosslinked protein crystals according to this invention. In such formulations, the crosslinked protein crystals are substantially insoluble under storage conditions and capable of releasing their protein activity in vivo at a controlled rate. For example, a pharmaceutical controlled release formulation according to this invention, administered by oral route, is characterized in that the component crosslinked protein crystals are substantially insoluble under gastric pH conditions and substantially soluble under small intestine pH conditions. Alternatively, for these and other uses according to this invention, the crosslinked protein crystals may be active in the insoluble form and then dissolve and are removed or digested once their function is complete.

Pharmaceutical, personal care, veterinary or prophylactic compositions comprising crosslinked protein crystals according to this invention may also be selected from the group consisting of tablets, liposomes, granules, spheres, microparticles, microspheres and capsules.

For such uses, as well as other uses according to this invention, crosslinked protein crystals may be formulated into tablets. Such tablets constitute a liquid-free, dust-free form of crosslinked protein crystal storage which are easily handled and retain acceptable levels of activity.

Alternatively, the crosslinked protein crystals may be in a variety of conventional depot forms employed for administration to provide reactive compositions. These include, for example, solid, semi-solid and liquid dosage forms, such as liquid solutions or suspensions, gels, creams, balms, emulsions, lotions, slurries, powders, sprays, foams, pastes, ointments, salves, balms and drops.

Compositions or formulations comprising the crosslinked protein crystals of this invention may also comprise any conventional carrier or adjuvant used in pharmaceuticals, personal care compositions or veterinary formulations. These carriers and adjuvants include, for example, Freund's adjuvant, ion exchangers, alumina, aluminum stearate, lecithin, buffer substances, such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts or electrolytes, such as protamine sulfate, disodium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium, trisilicate, cellulose-based substances and polyethylene glycol. Adjuvants for topical or gel base forms may include, for example, sodium carboxymethylcellulose, polyacrylates, polyoxyethylene-polyoxypropylene-block polymers, polyethylene glycol and wood wax alcohols.

According to one embodiment of this invention, crosslinked protein crystals may be combined

with any conventional materials used for controlled release administration. Such materials include, for example, coatings, shells and films, such as enteric coatings and polymer coatings and films.

The most effective mode of administration and dosage regimen of formulations or compositions comprising crosslinked protein crystals of this invention will depend on the effect desired, previous therapy, if any, the individual's health status or status of the condition itself and response to the crosslinked protein crystals and the judgment of the treating physician or clinician. The crosslinked protein crystals may be administered in any dosage form acceptable for pharmaceuticals, personal care compositions or veterinary formulations, at one time or over a series of treatments.

The amount of the crosslinked protein crystals that may be combined with carrier materials to produce a single dosage form will vary depending upon the particular mode of administration, formulation, dose level or dose frequency. A typical preparation will contain between about 0.01% and about 99%, preferably between about 1% and about 50%, crosslinked protein crystals (w/w).

Upon improvement of the individual's condition, a maintenance dose of crosslinked protein crystals may be administered, if necessary. Subsequently, the dosage or frequency of administration, or both, may be reduced as a function of the symptoms, to a level at which the improved condition is retained. When the condition has been alleviated to the desired level, treatment should cease. Individuals may, however, require intermittent

treatment on a long-term basis upon any recurrence of the condition or symptoms thereof.

An alternate embodiment of the present invention includes protein delivery systems comprising the crosslinked protein crystals disclosed herein. Such a system may be used to deliver proteins such as those included in cleaning agents, such as detergents, personal care products, such as cosmetics, pharmaceuticals, such as lipase, veterinary compositions, vaccines, foods, feeds, diagnostics and formulations for decontamination. Protein delivery systems of this invention, which may be formulations or devices, such as implantable devices, may be microparticulate protein delivery systems, wherein the crosslinked protein crystals have a longest dimension between about 0.01 μm and about 500 μm, alternatively between about 0.1 μm and about 50 μm. The crosslinked protein crystal components of such systems may have a shape selected from the group consisting of: spheres, needles, rods, plates, such as hexagons and squares, rhomboids, cubes, bipryamids and prisms. Advantageously, the crosslinked crystal form of the proteins of this invention allow loading of up to between about 50% and about 90% protein per unit of weight.

One example of a protected protein system according to this invention is suitable for storage in a medium such as a liquid detergent, prior to use. The crosslinked protein crystal components of such a system are insoluble under storage conditions in said medium -- which typically causes degradation of the soluble form of the protein that is crystallized to form said crystal that is crosslinked -- and soluble under conditions of use.

According to the present invention, preparation of crosslinked protein crystals includes the steps of crystallizing and crosslinking the protein. This may be carried out as illustrated below.

Preparation of Crosslinked Protein Crystals - Protein Crystallization

Protein crystals are grown by controlled crystallization of protein out of aqueous solution or aqueous solution-containing organic solvents. Conditions to be controlled include, for example, the rate of evaporation of solvent, the presence of appropriate co-solutes and buffers, pH and temperature. A comprehensive review of the various factors affecting the crystallization of proteins has been published by McPherson, Methods Enzymol., 114, pp. 112-20 (1985).

McPherson and Gilliland, J. Crystal Growth, 90, pp. 51-59 (1988) have compiled comprehensive lists of proteins and nucleic acids that have been crystallized, as well as the conditions under which they were crystallized. A compendium of crystals and crystallization recipes, as well as a repository of coordinates of solved protein and nucleic acid structures, is maintained by the Protein Data Bank at the Brookhaven National Laboratory [http//www.pdb.bnl.gov; Bernstein et al., J. Mol. Biol., 112, pp. 535-42 (1977)]. These references can be used to determine the conditions necessary for crystallization of a protein, as a prelude to the formation of an appropriate crosslinked protein crystal, and can guide the crystallization strategy for other proteins. Alternatively, an intelligent trial and error search strategy can, in most instances, produce suitable crystallization conditions for many proteins, provided

that an acceptable level of purity can be achieved for them [see, e.g., C.W. Carter, Jr. and C.W. Carter, J. Biol. Chem., 254, pp. 12219-23 (1979)].

For use in crosslinked protein crystals according to this invention, the large single crystals which are needed for X-ray diffraction analysis are not required. Microcrystalline showers are suitable.

For example, the crosslinked protein crystals may have a longest dimension between about 0.01 μm and about 500 μm, alternatively, between 0.1 μm and about 50 μm. They may also have a shape selected from the group consisting of: spheres, needles, rods, plates, such as hexagons and squares, rhomboids, cubes, bipryamids and prisms.

In general, crystals are produced by combining the protein to be crystallized with an appropriate aqueous solvent or aqueous solvent containing appropriate crystallization agents, such as salts or organic solvents. The solvent is combined with the protein and may be subjected to agitation at a temperature determined experimentally to be appropriate for the induction of crystallization and acceptable for the maintenance of protein activity and stability. The solvent can optionally include co-solutes, such as divalent cations, co-factors or chaotropes, as well as buffer species to control pH. The need for co-solutes and their concentrations are determined experimentally to facilitate crystallization.

In an industrial-scale process, the controlled precipitation leading to crystallization can best be carried out by the simple combination of protein, precipitant, co-solutes and, optionally, buffers in a batch process. As another option, proteins may be crystallized by using protein

precipitates as the starting material. In this case, protein precipitates are added to a crystallization solution and incubated until crystals form. Alternative laboratory crystallization methods, such as dialysis or vapor diffusion, can also be adopted. McPherson, supra and Gilliland, supra, include a comprehensive list of suitable conditions in their reviews of the crystallization literature.

Occasionally, incompatibility between the crosslinking agent and the crystallization medium might require exchanging the crystals into a more suitable solvent system.

Many of the proteins for which crystallization conditions have already been described, may be used to prepare crosslinked protein crystals according to this invention. It should be noted, however, that the conditions reported in most of the above-cited references have been optimized to yield, in most instances, a few large, diffraction quality crystals. Accordingly, it will be appreciated by those of skill in the art that some degree of adjustment of these conditions to provide a high yielding process for the large scale production of the smaller crystals used in making crosslinked protein crystals may be necessary.

Preparation of Crosslinked Protein Crystals - Crosslinking of Protein Crystals

Once protein crystals have been grown in a suitable medium they can be crosslinked. Crosslinking results in stabilization of the crystal lattice by introducing covalent links between the constituent protein molecules of the crystal. This makes possible transfer of the protein into an alternate environment

that might otherwise be incompatible with the existence of the crystal lattice or even with the existence of intact protein.

Advantageously, crosslinking according to the present invention is carried out in such a way that, under conditions of storage, the crosslinking interactions prevent the constituent protein molecules in the crystal from going back into solution, effectively insolubilizing or immobilizing the protein molecules into microcrystalline particles. Upon exposure to a trigger in the environment surrounding the crosslinked protein crystals, such as under conditions of use rather than storage, the protein molecules dissolve, releasing their protein activity. The rate of dissolution is controlled by one or more of the following factors: the degree of crosslinking, the length of time of exposure of protein crystals to the crosslinking agent, the amino acid residues involved in the crosslinks, whether the crosslinker is homobifunctional or heterobifunctional, the rate of addition of crosslinking agent to the protein crystals, the nature of the crosslinker, the chain length of the crosslinker, the surface area of the crosslinked protein crystals, the size of the crosslinked protein crystals, the shape of the crosslinked protein crystals and combinations thereof.

Alternatively, controlled dissolution crosslinked protein crystals function as crosslinked protein crystals in the absence of the specific trigger required for initiating and maintaining dissolution.

Crosslinking can be achieved using one or a combination of a wide variety of multifunctional reagents, at the same time (in parallel) or in sequence, including bifunctional reagents. Upon

exposure to a trigger in the surrounding environment, or over a given period of time, the crosslinks between protein crystals crosslinked with such multifunctional crosslinking agents lessen or weaken, leading to protein dissolution or release of activity. Such crosslinking agents include glutaraldehyde, succinaldehyde, octanedialdehyde and glyoxal. Additional multifunctional crosslinking agents include halo-triazines, e.g., cyanuric chloride; halo-pyrimidines, e.g., 2,4,6-trichloro/bromo-pyrimidine; anhydrides or halides of aliphatic or aromatic mono- or di-carboxylic acids, e.g., maleic anhydride, (meth)acryloyl chloride, chloroacetyl chloride; N-methylol compounds, e.g., N-methylol-chloro acetamide; di-isocyanates or di-isothiocyanates, e.g., phenylene-1,4-di-isocyanate and aziridines. Other crosslinking agents include epoxides, such as, for example, di-epoxides, tri-epoxides and tetra-epoxides. According to a preferred embodiment of this invention, the crosslinking agent is glutaraldehyde, used alone or in sequence with an epoxide. For a representative listing of other available crosslinking reagents see, for example, the 1996 catalog of the Pierce Chemical Company. Such multifunctional crosslinking agents may also be used, at the same time (in parallel) or in sequence, with reversible crosslinking agents, such as those described below.

Crosslinkers useful in various embodiments of this invention are (1) those which create covalent links from one cysteine side chain of a protein to another another cysteine side chain, (2) those which create covalent links from one lysine side chain of a protein to another, or (3) those which create covalent links from one cysteine side chain of a protein to a

lysine side chain. Crosslinking may occur through intermolecular and intramolecular covalent crosslinks.

Protein crystals may be crosslinked with one of the following crosslinkers to produce controlled dissolution crosslinked protein crystals: dimethyl 3, 3'-dithiobispropionimidate·HCl (DTBP); dithiobis (succinimidylpropionate) (DSP); bismaleimidohexane (BMH); bis[Sulfosuccinimidyl]suberate (BS); 1,5-difluoro-2,4-dinitrobenzene (DFDNB); dimethylsuberimidate·2HCl (DMS); disuccinimidyl glutarate (DSG); disulfosuccinimidyl tartarate (Sulfo-DST); 1-ethyl-3-[3-dimethylaminoproplyl]carbodiimide hydrochloride (EDC); ethylene glycolbis [sulfo-succinimidylsuccinate] (Sulfo-EGS); N-[γ-maleimido-butyryloxy]succinimide ester (GMBS); N-hydroxysulfo-succinimidyl-4-azidobenzoate (Sulfo-HSAB); sulfosuccinimidyl-6-[α-methyl-α-(2-pyridyldithio) toluamido]hexanoate (Sulfo-LC-SMPT); bis-[β-(4-azidosalicylamido) ethyl]disulfide (BASED); and NHS-PEG-Vinylsulfone (NHS-PEG-VS).

According to an alternate embodiment of this invention, crosslinking may be carried out using reversible crosslinkers, in parallel or in sequence. The resulting crosslinked protein crystals are characterized by a reactive multi-functional linker, into which a trigger is incorporated as a separate group. The reactive functionality is involved in linking together reactive amino acid side chains in a protein and the trigger consists of a bond that can be broken by altering one or more conditions in the surrounding environment (e.g., pH, temperature, or thermodynamic water activity). This is illustrated diagrammatically as:

$$X—Y—Z + 2\ AA\ residues \rightarrow AA_1-X—Y—Z-AA_2$$

change in environment --> AA_1-X+Y—Z-AA_2

-- where X and Z are groups with reactive functionality

-- where Y is a trigger

-- where AA_1 and AA_2 represent reactive amino acid residues on the same protein or on two different proteins. The bond between the crosslinking agent and the protein may be a covalent or ionic bond, or a hydrogen bond. The change in surrounding environment results in breaking of the trigger bond and dissolution of the protein. Thus, the crosslinks between protein crystals crosslinked with such reversible crosslinking agents break, leading to protein crystal dissolution or release of activity.

Alternatively, the reactive functionality of the crosslinker and the trigger may be the same, as in:

X—Z + 2AA residues --> AA_1-X—Z-AA_2

change in environment --> AA_1 + X—Z-AA_2.

The crosslinker may be homofunctional (X=Y) or heterofunctional (X is not equal to Y). The reactive functionality X and Y may be, but not limited to the following functional groups (where R, R', R", and R'" may be alkyl, aryl or hydrogen groups):

I. Reactive acyl donors are exemplified by: carboxylate esters RCOOR', amides RCONHR', Acyl azides $RCON_3$, carbodiimides R-N=C=N-R', N-hydroxyimide esters, RCO-O-NR', imidoesters R-C=$NH2^+$(OR'), anhydrides RCO-O-COR', carbonates RO-CO-O-R', urethanes RNHCONHR', acid halides RCOHal (where Hal=a halogen), acyl hydrazides RCONNR'R", O-acylisoureas RCO-O-C=NR'(-NR"R'"),

II. Reactive carbonyl groups are exemplified by: aldehydes RCHO and ketones RCOR', acetals $RCO(H_2)R'$, ketals $RR'CO_2R'R"$. Reactive carbonyl containing functional groups known to those well

skilled in the art of protein immobilization and crosslinking are described in the literature [Pierce Catalog and Handbook, Pierce Chemical Company, Rockford, Illinois (1994); S.S. Wong, Chemistry of Protein Conjugation and Cross-Linking, CRC Press, Boca Raton, Florida (1991)].

III. Alkyl or aryl donors are exemplified by: alkyl or aryl halides R-Hal, azides $R-N_3$, sulfate esters RSO_3R', phosphate esters $RPO(OR'_3)$, alkyloxonium salts R_3O+, sulfonium R_3S+, nitrate esters $RONO_2$, Michael acceptors RCR'=CR'"COR", aryl fluorides ArF, isonitriles RN+≡C-, haloamines R_2N-Hal, alkenes and alkynes.

IV. Sulfur containing groups are exemplified by disulfides RSSR', sulfhydryls RSH, epoxides $R_2C^{O}CR'_2$.

V. Salts are exemplified by alkyl or aryl ammonium salts R_4N+, carboxylate RCOO-, sulfate $ROSO_3-$, phosphate $ROPO_3$" and amines R_3N.

The table below includes examples of triggers, organized by release mechanism. In the table, R= is a multifunctional crosslinking agent that can be an alkyl, aryl, or other chains with activating groups that can react with the protein to be crosslinked. Those reactive groups can be any variety of groups such as those susceptible to nucleophilic, free radical or electrophilic displacement including halides, aldehydes, carbonates, urethanes, xanthanes, epoxides among others.

Trigger	Examples	Release Conditions
1. Acid Labile Linkers	R-O-R e.g. Thp, MOM, • Acetal, ketal • Aldol, Michael adducts, esters	H^+ or Lewis Acidic catalysts
2. Base Labile Linkers	• R'OCO2-R' Carbonates • R'O-$CONR_2$ Carbamates • R_2'$NCONR_2$ Urethanes • Aldol, Michael adducts, esters	Variety of basic media
3. Fluoride Labile Linkers	R-$OSiR_3$ Various Si containing linkers	Aqueous F^-
4. Enzyme Labile Linkers	• RCOOR, R$CONR_2$'	Free lipases, amidases, esterases
5. Reduction Labile Linkers	• Disulfide linkers that cleave via Hydrogenolysis • Reductive Elimination • R'-S-S-R	H_2 catalyst; Hydrides
6. Oxidation Labile Linkers	• R-$OSiR_3$ • Glycols R-CH(OH)-CH(OH)-R'	Oxidizing agents: e.g. H_2O_2, NaOCl, IO_4^- Metal based oxidizers, other hypervalent oxidents
7. Thio-labile linkers	• R'-S-S-R	Thiols, e.g., Cys, DTT, mercaptoethanol

Trigger	Examples	Release Conditions
8. Heavy Metal Labile Linkers	• Various Allyl Ethers $ROCH_2CH=CHR$ Alkyl, Acyl • Allyl ester	Transition metal based reagents (Pd, Ir, Hg, Ag, Cu, Tl, Rh) • Pd(0) catalysts
9. Photolabile Linkers	• O-nitrobenzyl (ONB) • DESYL groups in linker	light (hv)
10. Free Radical Labile Linkers	• Thiohydroxa-mate ester (Barton ester)	• Free radical initiator
11. Metal-chelate linked	• Iron (III) diphenanthroline	• Metal removal e.g. by chelation or precipitation
12. Thermally Labile Linkers	• Peroxides R-OO-R	• Increase in temperature
13. "Safety Catch" Labile Linkers	• Methylthio-ethyl (Mte) • Dithianes	Base; amines, others

The dissolution of the crosslinked protein crystals of this invention can be controlled by selecting appropriate crosslinkers and the associated triggers. Examples of physical and chemical triggers available include: change in temperature, change in pH, change in chemical composition, change from concentrate to dilute form, change in oxidation-reduction potential of the solution, change in the incident radiation, change in transition metal concentration, change in flouride concentration, change in free radical concentration, change in metal chelater concentration, change in shear force acting upon the crystal and combinations thereof.

Additional examples of reversible crosslinkers are described in T.W. Green, Protective Groups in Organic Synthesis, John Wiley & Sons (Eds.) (1981). Any variety of strategies used for reversible protecting groups can be incorporated into a crosslinker suitable for producing crosslinked protein crystals capable of reversible, controlled solubilization. Various approaches are listed, in Waldmann's review of this subject, in Angewante Chemie Inl. Ed. Engl., 35, p. 2056 (1996).

Other types of reversible crosslinkers are disulfide bond-containing crosslinkers. The trigger breaking crosslinks formed by such crosslinkers is the addition of reducing agent, such as cysteine, to the environment of the crosslinked protein crystals.

Disulfide crosslinkers are described in the Pierce Catalog and Handbook (1994-1995).

Examples of such crosslinkers include:

Homobifunctional (Symmetric)

DSP - Dithiobis(succinimidylpropionate), also know as Lomant's Reagent

DTSSP - 3-3'-Dithiobis(sulfosuccinimidylpropionate), water soluble version of DSP

DTBP - Dimethyl 3,3'-dithiobispropionimidate•HCl

BASED - Bis-(β-[4-azidosalicylamido]ethyl)disulfide

DPDPB - 1,4-Di-(3'-[2'-pyridyldithio]-propionamido)butane.

Heterobifunctional (Asymmetric)

SPDP - N-Succinimidyl-3-(2-pyridyldithio)propionate

LC-SPDP - Succinimidyl-6-(3-[2-pyridyldithio] propionate)hexanoate

Sulfo-LC-SPDP - Sulfosuccinimidyl-6-(3-[2-pyridyldlthio] propionate)hexanoate, water soluble version of LC-SPDP

APDP - N-(4-[p-azidosalicylamido]butyl)-3'-(2'-pyridyldithio) propionamide

SADP - N-Succinimidyl(4-azidophenyl)1,3'-dithiopropionate

Sulfo-SADP - Sulfosuccinimidyl(4-azidophenyl) 1,3'-dithiopropionate, water soluble version of SADP

SAED - Sulfosuccinimidyl-2-(7-azido-4-methycoumarin-3-acetamide)ethyl-1,3'dithiopropionate

SAND - Sulfosuccinimidyl-2-(m-azido-o-nitrobenzamido)ethyl-1,3'-dithiopropionate

SASD - Sulfosuccinimidyl-2-(p-azidosalicylamido)ethyl-1,3'-dithiopropionate

SMPB - Succinimidyl-4-(p-maleimidophenyl)butyrate

Sulfo-SMPB - Sulfosuccinimidyl-4-(p-maleimidophenyl)butyrate

SMPT - 4-Succinimidyloxycarbonyl-methyl-α-(2-pyridylthio) toluene

Sulfo-LC-SMPT - Sulfosuccinimidyl-6-(α-methyl-α-(2-pyridylthio)toluamido)hexanoate.

In order that this invention may be better understood, the following examples are set forth. These examples are for the purpose of illustration only and are not to be construed as limiting the scope of the invention in any manner.

EXAMPLES

Example 1 - Preparation of Crosslinked Subtilisin Crystals

Crystallization of Subtilisin

One volume of Alcalase 2.5L (Novo Nordisk Bioindustrials, Franklinton, North Carolina) was added to 2 volumes of a solution of 15% sodium sulfate (pH 5.5) prepared at 30-35°C. The crystallization solution was seeded with 1/2,000-1/500 volume seeds (30 mg/ml slurry of crystals in 15% sodium sulfate (pH 5.5), pH supported at 5.5 by adding NaOH. The seeded crystallization solution was incubated at 30-35°C, stirring by magnetic stirrer overnight. This yielded 60-80% (by activity) crystal rods, 10-50 µm, in length, 1-3 µm in width, after 24-48 hours.

Example 2 - Crosslinking of Subtilisin Crystals

Subtilisin crystals were crosslinked using one of a variety of crosslinkers, including: glutaraldehyde, glyoxal, succinaldehyde, octanedialdehyde and epoxides.

Glutaraldehyde Crosslinking

Glutaraldehyde ("GA") (supplied as 50% in aqueous by Aldrich Chemical Co.) was diluted in deionized water at 4°C in the various amounts listed in Table I below. For each ml of subtilisin crystals (27 mg/ml) in 15% sodium sulfate, 10 µl of the diluted glutaraldehyde was added to the slurry while shaking on a vortex at low speed (for amounts less than 5 ml) or stirring with an overhead stirrer at medium speed (for amounts 25 ml - 500 ml). After mixing for the allotted crosslinking time, the samples were centrifuged for 20

seconds at maximum speed, the supernatant was discarded and replaced with 15% sodium sulfate. This "washing" was repeated a total of 5 times. The final resuspension was effected with 900 µl of 15% sodium sulfate.

Table I - Glutaraldehyde Crosslinking

% GA-final	GA(ml)	H_2O(ml)	Cross-linking time(min)
0.0076	1.0	64.96	60
0.0189	1.0	25.46	39
0.02	1.0	24.0	39, 81
0.05	1.0	9.00	15, 60, 89
0.08	1.0	5.25	39, 81
0.10	1.0	4.00	60, 81
0.125	1.0	3.00	3, 10, 17, 39
0.15	1.0	2.33	81, 120
0.20	1.0	1.50	19, 60, 120
0.231	1.0	1.16	10, 39, 120
0.3	1.0	0.67	60
0.5	1.0	0	60

Glyoxal Crosslinking

Glyoxal (supplied as 40% in aqueous by Aldrich Chemical Co.) ("GY") was added to the crystal suspension to give a final concentration of 0.01-1.0%. For each ml of subtilisin crystals (27 mg/ml) in 15% sodium sulfate 0.25 µl to 25 ml (0.01 to 1%) of the glyoxal was added to the slurry, while magnetically

stirring at ambient temperature. After stirring for 1 hour, the crosslinked crystals were centrifuged and washed, as described for glutaraldehyde crosslinking.

Octanedialdehyde Crosslinking

Octanedialdehyde ("OA") (100% as supplied by DSM Chemie Linz), in the amounts shown in Table II below, was added undiluted to 1 ml of subtilisin crystal slurry (27 mg/ml in 15% sodium sulfate) while magnetically stirring at ambient temperature. Stirring was continued for the specified time of minutes or hours before the crosslinked crystals were centrifuged and washed, as described for glutaraldehyde crosslinking.

Table II - Octanedialdehyde Crosslinking

% OA - final	OA (µl)	Crosslinking time
0.05	0.5	16h
0.1	1.0	16h
0.2	2.0	16h
0.25	2.5	16h
0.5	5.0	16h
1.0	10.0	30m, 1h, 3h, 16h

Succinaldehyde Crosslinking

Succinaldehyde ("SA")(40% as supplied by DSM Chemie Linz) was added undiluted, in the amounts shown in Table III below, to 1 ml of subtilisin crystal slurry (27 mg/ml in 15% sodium sulfate) while magnetically stirring at ambient temperature. Stirring was continued for the specified time of minutes or hours before the crosslinked crystals were centrifuged

and washed, as described for glutaraldehyde crosslinking.

Table III - Succinaldehyde Crosslinking

%SA - final	SA (µl)	Crosslinking time
1.0	25	30m, 1h, 3h

Epichlorohydrin Crosslinking

A 10 µl aliquot of epichlorohydrin ("EP") (99%, Sigma Chemical Co., St. Louis, Missouri) was added undiluted to 1 ml of subtilisin crystal slurry (27 mg/ml in 15% sodium sulfate) while stirring at ambient temperature. Stirring was continued for the specified time of minutes or hours before the crosslinked crystals were centrifuged and washed, as described for glutaraldehyde crosslinking.

Epoxide Crosslinking

General Procedure

Crosslinking of subtilisin was carried out individually using one of a variety of epoxides. These included:

1) General name - DENACOL

a) DENACOL EX-411
b) DENACOL EX-421
c) DENACOL EX-614
d) DENACOL EX-201
e) DENACOL EX-202; all obtained from Nagase American Corporation.

2) Obtained from Tokyo Kasei Inc. America:

a) Neopentyl Glycol diglycidyl Ether (N448) ("NP")
b) Ethylene Glycol diglycidyl Ether (EO342) ("EG").

The concentration of the epoxide was varied between 0.01 and 4.0% and the crosslinking time was varied from 1 hour to 72 hours. The procedure for addition to and removal of crosslinker from enzyme was as described above for glutaraldehyde crosslinking.

Subsequent crosslinking with glutaraldehyde (0.01 to 0.2%) for (1 hour to 5 hours) yielded strongly crosslinked enzyme crystals, insoluble in water, but active in the azocasein assay.

A sample of 1 ml of subtilisin crystal slurry (27 mg/ml in 15% sodium sulfate) was mixed by vortexing at low speed to assure a uniform suspension of crystals. Epoxide (10% solution in DMF) was added to the crystal slurry in the amounts specified in Table IV, and the mixture was shaken at ambient temperature. After the allotted time between 1 and 72 hours at ambient temperature, glutaraldehyde (10% in DMF) was added to the epoxide/crystal mixture and stirring was continued at ambient temperature for the time specified in Table IV. The resulting crosslinked enzyme crystals were washed 2x with 1% $(NH_4)_2SO_4$/10mM $CaCl_2$ then 3x with water and finally 1x with 1% $(NH_4)_2SO_4$/10mM $CaCl_2$ before resuspending in 1% $(NH_4)_2SO_4$/10mM $CaCl_2$.

Table IV - Epoxide/Glutaraldehyde Crosslinking

Epoxide Name	Epoxide Amount	Epoxide Crosslinking Time	Glutaraldehyde Amount	Glutaraldehyde Crosslinking Time
EX-411	0.01-4%	1-72h	0.01-0.1%	0.5-2h
EX-421	0.01-4%	1-72h	0.01-0.1%	0.5-2h
EX-614	0.01-4%	1-72h	0.01-0.1%	0.5-2h
EX-201	0.01-4%	1-72h	0.01-0.1%	0.5-2h
EX-202	0.01-4%	1-72h	0.01-0.1%	0.5-2h

Epoxide Name	Epoxide Amount	Epoxide Crosslinking Time	Glutaraldehyde Amount	Glutaraldehyde Crosslinking Time
NP (N448)	0.01-4%	1-72h	0.01-0.1%	0.5-2h
EG (EO342)	0.01-4%	1-72h	0.01-0.1%	0.5-2h

Large Scale Preparation of A Preferred Epoxide Sample

Prior to crosslinking, a sample of 380 ml of crystalline subtilisin in 15% sodium sulfate (27 mg/ml) was mixed by overhead stirring at ambient temperature for 5 minutes to assure a uniform suspension of crystals. Neopentyl glycol diglycidyl ether (3.838 ml of a 10% solution in DMF) was added to the crystal slurry and the mixture was stirred at ambient temperature. After 5 hours at ambient temperature, 3.838 ml of glutaraldehyde (10% in DMF) was added to the epoxide/crystal mixture and stirring was continued at ambient temperature for 1.5 hours. The resulting crosslinked enzyme crystals were washed 2x with 1% $(NH_4)_2SO_4$/10mM $CaCl_2$ then 3x with water and finally 1x with 1% $(NH_4)_2SO_4$/10mM $CaCl_2$, before resuspending in 1% $(NH_4)_2SO_4$/10mM $CaCl_2$.

Example 3 - Activity Assay

In order to test the activity of crosslinked protein crystals according to this invention, as well as other enzyme samples, we developed the following azocasein assay.

Materials:

2.0M Tris Buffer. 500ppm $CaCl_2$

0.2M Tris Buffer. 50ppm $CaCl_2$

50% urea

Azocasein

5% trichloroacetic acid ("TCA")
Alcalase (2.5L)
ChiroCLEC-BL™ (crosslinked subtilisin crystals, available from Altus Biologics, Inc., Cambridge, Massachusetts)

The assay was carried out, preparing azocasein just prior to use, by dissolving 600 mg azocasein with 10 ml of 50% urea and vortexing lightly to complete the dissolution. Then 10 ml 2.0M Tris was added and vortexed to mix, increasing the volume to 100 ml by adding deionized water.

The stock solutions of the enzyme to be assayed in 0.2M Tris were prepared, to provide 50 μl aliquots to be assayed, as follows:

Without detergent: 0.03 mg/ml Alcalase (soluble, uncrosslinked subtilisin Carlsberg 80.3 mg/ml)
3.0 mg/ml ChiroCLEC-BL™.

With 120 μl detergent/ml solution:
0.03 mg/ml Alcalase
3.0 mg/ml ChiroCLEC-BL™.

We added 50 μl aliquots of enzyme to 150 μl of 0.2M Tris and placed the mixtures in 5 ml test tubes with micro-stir bars. We then warmed both the test tubes and the azocasein at 40°C for 1 minute using a metal heating block. After that, we added 1 ml of the azocasein to each tube and stirred at 40°C for 15 minutes using the heating block at stir speed 4. We then added 2 ml TCA to each tube, mixing by vortex, and placed the tubes in an ice bath immediately, allowing

the samples to stand at 0°C for 20 minutes. We microfuged the samples for 5 minutes at maximum rpm and microfiltered, if necessary. We measured absorbance of the expressed activity in abs·units/mg protein·min supernatant at λ390. In this assay, all measurements were done in triplicate. Controls were void of enzyme but contained detergent if it was present in the assay. This time = 0 assay was repeated at time = 15 minutes and other times, if necessary.

The detergents used in the various assays included Tide, Wisk and Ciba-Geigy detergents #15, #16 and #44 ("Ciba detergents"). Ciba detergent #15 constitutes a typical European detergent formulation -- liquid (aqueous) detergent on the basis of 15% alkylbenzene sulfonate, 14% fatty alcohol ethoxylate and 10% fatty acid salt (soap). Ciba detergent #16 constitutes a typical United States detergent formulation -- liquid (aqueous) detergent on the basis of 7.5% alkyl benzene sulfonate, 10% fatty alcohol ethoxylate and 17% alkyl either sulfate. Ciba detergent #44 constitutes a typical compact detergent formulation -- liquid (aqueous) detergent on the basis of 6% fatty alcohol ethoxylate, 23% alkyl ether sulfate and 10% sodium citrate. Ciba detergents #15, #16 and #44 may be obtained upon request from Ciba Specialty Chemicals Corp., Division Consumer Care Chemicals, Greensboro, North Carolina.

We prepared assay stock solutions from dilution stocks, and carried out the assays, as follows.

Activity Assay - 200x Dilution - Crosslinked Subtilisin Crystals and Crystalline Subtilisin in Heavy Duty Liquid Detergent (Ciba #15, Ciba #44, Tide and Wisk)

Stocks A, B, C and D were prepared in 10 ml neoprene tubes as follows.

Stock A : Crosslinked Subtilisin Crystals Prepared According to this Invention (~27 mg/ml)

We centrifuged 37 μl slurry of crosslinked subtilisin crystals (equal to 1 mg crosslinked enzyme crystals) to remove supernatant, added 1 ml detergent and vortexed to mix. A 50 μl aliquot of the resulting mixture was added to 9.95 ml water, to a final concentration of 5 μg/ml.

Stock B : Uncrosslinked Subtilisin Crystals (~27 mg/ml)

We centrifuged 37 μl slurry of subtilisin crystals (equal to 1 mg enzyme crystals) to remove supernatant, added 1 ml detergent and vortexed to mix. A 50 μl aliquot of the resulting mixture was added to 9.95 ml water, to a final concentration of 5 μg/ml.

Stock C: Alcalase

We added 18.75 μl Alcalase (80.3 mg/ml) to 3 ml detergent and vortexed to mix. A 50 μl aliquot of the resulting mixture was added to 9.95 ml water, to a final concentration of 2.5 μg/ml.

Stock D : Detergent

A 50 μl aliquot of detergent was added to 9.95 ml water.

Azocasein stock (6 mg/ml) was prepared as described above. Upon dilution of Stock A and B to 5 μg/ml, the t=0 assay was set up immediately and carried out as described above, except that the amount of stock sample used was 200 μl, instead of 50 μl + 150 μl 0.2M Tris. While the tubes were heating for 1 minute at 40°C, two additional samples of 2 ml each of Stocks A, B and C were placed in 1.5 ml microcentrifuge tubes and heated to 52°C while shaking for further testing after 5 minutes and 15 minutes dilution with heating.

Activity Assay - 670x Dilution -
Crosslinked Subtilisin Crystals and Crystalline Subtilisin in Detergent Concentrate (Ciba #16)

Stocks A, B, C and D were prepared in 10 ml neoprene tubes as follows.

Stock A : Crosslinked Subtilisin Crystals Prepared According to this Invention (~27 mg/ml)

We centrifuged 124 μl slurry of crosslinked subtilisin crystals (equal to 3.35 mg crosslinked enzyme crystals) to remove supernatant, added 1 ml detergent and vortexed to mix. A 50 μl aliquot of the resulting mixture was added to 33.45 ml water, to a final concentration of 5 μg/ml.

Stock B : Uncrosslinked Subtilisin Crystals (~27 mg/ml)

We centrifuged 124 μl slurry of subtilisin crystals (equal to 3.35 mg enzyme crystals) to remove supernatant, added 1 ml detergent and vortexed to mix. A 50 μl aliquot of the resulting mixture was added to 33.45 ml water, to a final concentration of 5 μg/ml.

Stock C: Alcalase

We added 167 μl Alcalase (80.3 mg/ml) to 8 ml detergent and vortexed to mix. A 50 μl aliquot of the resulting mixture was added to 33.45 ml water, to a final concentration of 2.5 μg/ml.

Stock D : Detergent

A 50 μl aliquot of detergent was added to 33.45 ml water.

Azocasein stock (6 mg/ml) was prepared as described above. The t=0 assay was set up immediately and carried out as described above, except that the amount of stock sample used was 200 μl, instead of 50 μl, plus 150 μl 0.2M Tris buffer. While the tubes were heating for 1 minute at 40°C, two additional samples of 2 ml each of Stocks A, B and C were placed in Eppendorf tubes and heated to 40°C while shaking for further testing after 5 minutes and 15 minutes dilution with heating.

Example 4 - Stability Study

In order to test the stability of crosslinked enzyme crystals according to this invention, as well as other enzyme samples, we developed the following assays.

Azocasein Assay - Stability Study 52°C

First, we prepared stock solutions of the enzyme samples in detergent in 2 ml Eppendorf tubes with screw caps. After incubating the mixtures in a water bath at 52°C for the appropriate times, we added 1.47 ml of 0.2M Tris buffer to one of each enzyme

sample tube, and mixed well. To assay for activity after the appropriate time of incubation followed by dilution, we removed a 50 μl aliquot from each tube and assayed as described below. The remaining samples of enzyme/detergent stocks were placed in a water bath at 52°C, with further aliquots being removed for assay at specific times.

The assay was performed by adding 50 μl enzyme sample to 150 μl 0.2M Tris buffer and heating to 40°C for 1 minute. At a constant 40°C temperature, we then added 1.0 ml azocasein stock (as described in Example 1) to each sample, stirring for 15 minutes using a heating block at stir speed 4. We then added 2 ml TCA to each tube, mixing by vortex, and placed the tubes in an ice bath immediately, allowing the samples to stand at 0°C for 20 minutes. We microfuged the samples for 5 minutes at maximum rpm and microfiltered, if necessary. We measured absorbance of the supernatant at λ390 and expressed activity as abs·units/mg protein·min. In this assay, all measurements were done in triplicate. Controls were void of enzyme but contained detergent if it was present in the assay.

In order to assess activity as part of stability studies carried out at 52°C, we prepared assay stock solutions from dilution stocks, and carried out the assays, as follows.

For Alcalase

Stocks:

Stock A: Alcalase (80.3 mg/ml) in commercial detergent (Tide or Wisk, deactivated by heating at 70°C for 4 hours) - final concentration = 0.25 mg/ml. The stock

was prepared by adding 31.2 μl Alcalase to 9.97 ml detergent. A 200 μl aliquot of the resulting mixture was placed in each of several 2 ml Eppendorf tubes (3x for t=0, 30 and others).

Stock B: Alcalase (80.3 mg/ml) in Ciba detergent (Ciba #15, Ciba #16 or Ciba #44) - final concentration = 0.25 mg/ml. The stock was prepared by adding 31.2 μl Alcalase to 9.97 ml detergent. A 200 μl aliquot of the resulting mixture was placed in each of several 2 ml Eppendorf tubes (3x for t=0, 1 hour and 4-6 hours).

Stock C: Commercial detergent (Tide or Wisk, deactivated by heating at 70°C for 4 hours) 3x (200 μl of the above in 2 ml Eppendorf tubes).

Stock D: Ciba detergent (Ciba #15, Ciba #16 or Ciba #44) 3x (200 μl of the above in 2 ml Eppendorf tubes).

The t=0 assay was performed immediately after 1.47 ml of 0.2 M Tris was added to one of each of tubes containing Stocks A-D and the contents mixed well. Remaining samples of Stocks A-D were placed in a water bath and heated to 52°C. Otherwise, the assays were carried out as described above.

For Crosslinked Subtilisin Crystals and Crystalline Subtilisin

Stocks A, B, C and D were prepared in 2 ml Eppendorf tubes with screw caps as follows.

Stock A: ChiroCLEC-BL™ in commercial detergent (denatured Tide or Wisk) - final concentration = 25 mg/ml. The stock was prepared by centrifuging 3.12 ml

enzyme slurry to remove water and then diluting the enzyme to 10 ml with detergent. A 200 μl aliquot of the resulting mixture was placed in each of several 2 ml Eppendorf tubes (4x for t=0, 24 hours, 48 hours and 72 hours).

Stock B: ChiroCLEC-BL™ in Ciba detergent (Ciba #15, Ciba #16 or Ciba #44)- final concentration = 25 mg/ml. The stock was prepared by centrifuging 3.12 ml enzyme slurry to remove water and then diluting the enzyme to 10 ml with detergent. A 200 μl aliquot of the resulting mixture was placed in each of several 2 ml Eppendorf tubes (4x for t=0, 24 hours, 48 hours and 72 hours at 52°C).

Stock C: Commercial detergent (Tide or Wisk, deactivated by heating at 70°C for 4 hours) 4x (200 μl of the above in 2 ml Eppendorf tubes).

Stock D: Ciba-Geigy detergent (Ciba #15, #16 or #44, depending on which detergent was chosen for Stock B) 4x (200 μl of the above in 2 ml Eppendorf tubes).

The t=0 assay was set up immediately after 1.47 ml of 0.2M Tris was added to one of each of tubes containing Stocks A-D and the contents mixed well. Remaining samples of Stocks A-D were placed in a water bath and heated to 52°C. Otherwise, the assays were carried out as described above.

For Crosslinked Subtilisin Crystals and Crystalline Subtilisin

Stocks A, B and C were prepared in 2 ml Eppendorf tubes with screw caps as follows.

Stock A: Uncrosslinked subtilisin crystals (~ 27 mg/ml) in Ciba detergent (Ciba #15, #16 or #44) - final concentration = ~ 1 mg/ml. The stock was prepared by centrifuging 50 µl crystal slurry to remove supernatant, then adding 1.35 ml detergent (Ciba #15, Ciba #16 or Ciba #44) to a final concentration of 1 mg/ml. An 80 µl aliquot of the resulting mixture was placed in each of several 2.0 ml Eppendorf tubes (3x for t=0, 15 minutes and others).

Stock B: Crosslinked subtilisin crystals according to this invention (~ 27 mg/ml) in Ciba detergent (Ciba #15, #16 or #44) - final concentration = ~ 1 mg/ml. The stock was prepared by centrifuging 50 µl crystal slurry to remove supernatant, then adding 1.35 ml detergent, to a final concentration of 1 mg/ml. An 80 µl aliquot of the resulting mixture was placed in each of several 2.0 ml Eppendorf tubes (3x for t=0, 15 minutes and others).

Stock C: Ciba detergent (Ciba #15, #16 or #44) - 3x (80 µl of the above in 2.0 ml tubes).

The t=0 assay was set up immediately after 1.8 ml of water was added to one of each of tubes containing Stocks A-C and the contents mixed well. Remaining samples of Stocks A-C were placed in a water bath and heated to 52°C. Otherwise, the assays were carried out as described above, except for the addition of 150 µl 0.2 M Tris buffer instead of 200 µl.

Azocasein Assay - Stability Study 40°C

First, we prepared stock solutions of the enzyme samples in detergent in 2 ml Eppendorf tubes with screw caps. To assay stability at t=0, we added 1.8 ml of deionized water to a 25 μl of each sample and mixed well. We removed a 25 μl aliquot from each tube and assayed as described below. The remaining samples of enzyme/detergent stocks were placed in a water bath at 40°C, with further aliquots being removed for assay at specific times.

The assay was performed by adding 25 μl of the diluted enzyme sample to 175 μl 0.2M Tris buffer and heating to 40°C for 1 minute. At a constant 40°C temperature, we then added 1.0 ml azocasein stock (as described in Example 3) to each sample, stirring for 15 minutes using a heating block at stir speed 4. We then added 2 ml TCA to each tube, mixing by vortex, and placed the tubes in an ice bath immediately, allowing the samples to stand at 0°C for 20 minutes. We microfuged the samples for 5 minutes at maximum rpm and microfiltered, if necessary. We measured absorbance of the supernatant at λ390 and expressed activity as abs·units/mg protein·min. In this assay, all measurements were done in triplicate. Controls were void of enzyme but contained detergent if it was present in the assay.

In order to assess activity as part of stability studies carried out at 40°C, we prepared assay stock solutions from dilution stocks, and carried out the assays, as follows.

Stock A : Crosslinked Subtilisin Crystals According to this Invention (~27 mg/ml)

We centrifuged 124 μl slurry of crosslinked subtilisin crystals (equal to 3.35 mg crosslinked enzyme crystals) to remove supernatant, added 1 ml detergent and vortexed to mix, to a final concentration of 3.35 mg/ml.

Stock B : Uncrosslinked Subtilisin Crystals (~27 mg/ml)

We centrifuged 124 μl slurry of subtilisin crystals (equal to 3.35 mg enzyme crystals) to remove supernatant, added 1 ml detergent and vortexed to mix, to a final concentration of 3.35 mg/ml.

Stock C: Alcalase

We added 20.9 μl Alcalase (80.3 mg/ml) to 1 ml Ciba detergent (Ciba #15, Ciba #16 or Ciba #44) and commercial detergent (Tide or Wisk, denatured by heating at 70°C for 4 hours) and vortexed to mix, to a final concentration of 1.67 mg/ml.

Stock D : Detergent

One ml of commercial detergent (Tide or Wisk, deactivated by heating at 70°C for 4 hours) and Ciba detergents #15, #16 and #44. A 25 µl aliquot of each stock was added to 1.8 ml of deionized water and mixed well. A further 25 µl aliquot of the diluted stock was added to each reaction tube.

The t=0 assay was performed immediately after 175 μl of 0.2M Tris was added to each tube containing 25 μl of the various stock samples. Remaining samples of Stocks A-D were placed in a water bath and heated to 40°C. Otherwise, the assays were carried out as described above.

Example 5 - Dissolution Studies

We also assessed the characteristics of crosslinked enzyme crystals according to this invention, as well as other enzyme samples, with respect to dissolution in concentrate and upon dilution, as detailed below. Stock solutions were prepared and diluted as described above. The resulting dispersions were heated at 40°C and analyzed under a microscope at 250x for dissolution progress.

Example 6 - Results of Activity and Stability Assays

Crosslinked enzyme crystals of subtilisin, as described above, as well as soluble enzymes and other commercial enzymes, alone and in the presence of commercial detergents, were tested for activity in the azocasein assay, as described above. Catalyst concentrations for equivalent activities were determined for Alcalase, ChiroCLEC-BL™, Wisk with active protease and Tide with active protease:

ChiroCLEC-BL™: 150 μg/6 μl detergent 0.4 - 0.5 absorbance units

Alcalase: 15 μg/6 μl detergent 0.5 - 0.6 absorbance units

Tide: 6 μl detergent approximately 0.6 absorbance units

Wisk: 6 μl detergent approximately 0.6 absorbance units.

The dilution studies (discussed supra) were started by assessing the activities of Alcalase and uncrosslinked crystals of Alcalase in Ciba detergents #15 and #16. Initial activities were comparable and

losses of up to ~ 50% were seen after 15 minutes at 52°C.

Table V summarizes the stability of samples of Alcalase (0.25 mg/ml) and ChiroCLEC-BL™ (25 mg/ml) in denatured Wisk or Tide detergent, or in Ciba detergents #15 and #16 at 52°C. Activity was measured by the azocasein assay.

TABLE V

Stability of Subtilisin in Detergents at 52°C

Detergent	Alcalase $T_{1/2}$ at 52°C	ChiroCLEC-BL™ $T_{1/2}$ at 52°C
#15	less than 15 min	»100 hours
#16	less than 15 min	»100 hours
Tide (denatured)	16 hours	»100 hours
Wisk (denatured)	60-70 hours	»100 hours

We also assessed the stability of various enzymes in Ciba detergent #15 at 52°C. The results are depicted in Table VI below:

TABLE VI

Stability of Subtilisin in Ciba Detergent #15 at 52°C

Catalyst	Initial Activity	Activity 15 min dilute at 52°c	$T_{1/2}$ in concentrate at 52°C
Alcalase	36	14	~15min
Alcalase	33	13	~15min
OA 0.1%, 16h	34	18	~15min
OA 1%, 1h	23	17	~30min
OA 1%, 3h	10	14	~30min

Catalyst	Initial Activity	Activity 15 min dilute at 52°c	$T_{1/2}$ in concentrate at 52°C
GA 0.05%, 30min	27	16	~40min
GA 0.05%, 10 min	35	15	~40min

All of the crosslinked crystals prepared as described in the table above which had half-lives in detergent concentrate of ~30 minutes or more also had good solubility profiles.

In addition, we assessed the stability of various enzymes in Ciba detergent #15 versus Ciba detergent #16 at 40°C. The results are depicted in the Table VII below:

TABLE VII

Stability of Subtilisin in Ciba Detergent #15 vs. #16 at 40°C

Catalyst	Initial Activity	$T_{1/2}$ in #15 concentrate at 40°C	$T_{1/2}$ in #16 concentrate at 40°C
Alcalase	33	10h	2.5h
GA 0.05%, 30min	27	7h	~10h
OA 0.1%, 16h	32	9h	8h
OA 0.2%, 16h	15	12h	16h

We also assessed the effects of crosslinking time on activity and stability of the resulting crosslinked enzyme crystals. These results are

summarized in the tables below. In Table VIII, an asterisk indicates values measured by incubating 25 μl in a 2 ml tube and "Xs" denotes uncrosslinked protein crystals.

In preparing the crosslinked protein crystals described in Tables VIII, IX and X, the protein crystals were crystallized as described in Example 1 and crosslinked with glutaraldehyde as described in Example 2, using the crosslinking times and glutaraldehyde concentrations set forth in that example, or those specified in the tables.

TABLE VIII

Crosslinking Time vs. Concentration of Glutaraldehyde on Stability of Subtilisin in Ciba Detergent #16 at 40°C

GA (%)	Cross-linking Time	Activity abs/mg/min t=0	Activity abs/mg/min t=18h	Stability, 18h % of Xs, t=0
(Xs) 0	0	33.6	1.1	3.3
(Xs) 0	0	31.7	2.6*	8.2*
0.0189	10.0	28.5	5.2	16.5
0.0189	10.0	31.3	5.7	17.8
0.0189	39.3	14.1	5.4	17.0
0.0189	39.3	14.3	5.0	15.8
0.05	5.0	20.7	3.8	12.0
0.05	15.0	16.4	7.0	22.1
0.05	18.6	19.6	8.7	27.4
0.05	18.6	17.8	9.8	30.9
0.05	60.0	0	13.5	42.6
0.05	60.0	3.0	14.7	46.4
0.125	3.0	18.3	9.1	28.7
0.125	3.0	15.4	9.0	28.4

GA (%)	Cross-linking Time	Activity abs/mg/min t=0	Activity abs/mg/min t=18h	Stability, 18h % of Xs, t=0
0.125	10.0	7.9	14.9	46.9
0.125	10.0	9.5	14.8	46.6
0.125	10.0	7.9	14.7	46.4
0.125	10.0	9.5	13.4	42.1
0.125	10.0	9.4	12.2	38.5
0.125	10.0	8.1	12.2	38.5
0.125	10.0	8.3	16.0	50.5
0.125	17.0	5.4	14.0	44.2
0.125	17.0	6.4	15.3	48.3
0.125	39.3	2.1	3.3	10.4
0.125	39.3	1.0	5.8	18.3
0.125	39.3	1.1	4.4	13.9
0.125	39.3	1.7	4.5	14.3
0.125	39.3	1.6	5.7	18.0
0.125	39.3	0.9	3.3	10.4
0.125	68.6	1.3	3.1	9.9
0.2	5.0	10.4	12.1	38.2
0.2	15.0	2.5	9.0	28.4
0.2	18.6	1.8	6.9	21.8
0.2	18.6	0.8	3.1	9.8
0.2	60.0	0.4	1.3	4.1
0.2	60.0	1.4	1.4	4.4
0.231	10.0	2.7	13.0	41.0
0.231	10.0	4.8	11.7	37.0
0.231	39.3	0.5	1.1	3.5
Alcalase		28.0	3.1*	9.8*
Alcalase		32.9	0.2	1.0

In Table IX, an asterisk indicates that crystals were crushed during crosslinking and dash marks indicate that no measurements were taken at those

points. All samples were prepared at 1 ml (27 mg) scale.

TABLE IX

Activity of Glutaraldehyde Crosslinked Subtilisin in Ciba Detergent #16 at 40°C

Crosslinking time		Activity at 40°C (abs/mg/min)						
GA(%)	(min)	t=0	18h	39h	63h	80h	90h	6days
(Xs)0	0	33.6	1.1	--	--	--	--	--
0.0076	60	14.1	3.0	--	--	--	--	--
0.02	39	12.0	7.7	--	--	--	--	--
0.02	80	6.9	11.5	--	--	--	0	--
0.02*	80	12.2	26.2	10.7	3	--	--	--
0.05*	31	13.5	26.3	9.8	5.3	--	--	--
0.05	60	4.9	17.7	7.9	2.4	--	--	1.0
0.05*	60	8.7	27.3	12.5	7.3	--	--	--
0.05	89	1.3	12.1	--	--	--	3.7	--
0.08	39	2.2	12.6	--	--	--	5.3	--
0.08	81	0.8	3.9	--	--	5.8	--	3.9
0.08*	81	9.8	--	--	--	10.2	--	--
0.125	3	16.4	9.7	1.7	0.3	--	--	--
0.125	10	9.4	14.8	9.4	1.9	--	--	--
0.125	17	6.4	15.3	11.5	8.5	4.6	--	--
0.2	5	10.4	12.7	5.1	0.3	--	--	--
0.23	10	4.8	11.7	10.0	8.5	5.2	--	--
Alcalase		30.5	1.6					

TABLE X

Conditions for Larger Scale Crosslinked Enzyme Crystal Preparation - Stability of Glutaraldehyde Crosslinked Subtilisin in Ciba Detergent #16 at 40°C

Crosslinking time		Stability at 40°C (abs/mg/min)				
GA(%)	(min)	t=0	18h			
(Xs)	0	33.9	--			
		t=0	16h	38h	59h	110h
*0.05	60	23.2	16.8	5.9	1.9	5.6
*0.08	80	14.4	12.2	4.1	2.4	--
*0.1	80	8.0	13.6	5.5	3.1	1.4
*0.125	60	9.3	20.9	13.6	--	2.3
*0.15	80	3.8	11.2	7.4	5.2	2.8
*0.231	60	5.2	9.9	9.3	6.6	8.3
*1.0	60	1.3	2.5	2.2	1.1	2.4

		t=0	24h	48h	72h	120h	168h	264h
§ 0.25	120	4.8	9.5	7.7	6.7	5.7	4.3	4.4
§ 0.20	120	2.6	9.5	8.6	8.7	5.6	--	4.5
§ 0.15	120	5.2	14.3	9.6	5.6	3.7	--	1.2
§ 0.1-NP/ 0.1 GA	5h/1.5h	9.6	10.2	6.3	--	--	5.7	

In Table X, an asterisk indicates that crosslinkings were carried out at a 1-2 g scale, § indicates that crosslinkings were carried out at a 10 g scale on previously crushed crystals and dash marks indicate that no measurements were taken at those points.

Figure 1 graphically depicts the stability of 10 g scale preparations of crosslinked subtilisin

crystals according to this invention in Ciba detergent #16 at 40°C. In Figure 1, "Altus I" represents crystals crosslinked with 0.25% glutaraldehyde for 2 hours; "Altus II" represents crystals crosslinked with 0.20% glutaraldehyde for 2 hours; "Altus III" represents crystals crosslinked with 0.15% glutaraldehyde for 2 hours and "Altus IV" represents crystals crosslinked with 0.1% neopentyl glycol diglycidyl ether for 5 hours, followed by 0.1% glutaraldehyde for 1.5 hours. All the crosslinked samples were crushed prior to crosslinking using a Brinkman Polytron Homogenizer, then prepared on a 10 g scale and monitored by the azocasein assay over one week at 40°C.

Example 7 - Results of Dissolution Study

The dissolution study demonstrated whether various crosslinked enzyme crystals dissolve in concentrate and the extent to which they dissolve upon dilution under conditions of use, for example under wash conditions. Representative results of this test are included in the tables below, in which "+" indicates that the sample dissolved, "-" indicates that the sample did not dissolve, "-/+" indicates that the sample dissolved somewhat (1 mg/ml in detergent liquid). In the tables, "GP" denotes crystals crosslinked as described infra for GA crosslinking using, instead, ultrapure glutaraldehyde (supplied as an 8% aqueous solution by the Sigma Chemical Co.) which was not diluted prior to addition to the protein crystals.

TABLE XI

Detergent Liquid Incubation Study -

Dissolution Study - Concentrate 14h at 40°C

Catalyst	Ciba #15	Ciba #16	Ciba #44	Tide
OA 1%, 16h	-	-/+	+	-
OA 0.5%, 16h	-	-	+	-
OA 0.1%, 16h	-	+	+	-
OA 0.2%, 16h	-	-/+	+	-
GA 0.5%, 1h	-	-/+	+	-
GA 0.9%, 1h	-	-	-	-
GA 0.7%, 1h	-	-	+	-
EP 1.0%,20min	-/+	+	+	-
GP 0.08%,20min	-	-/+	+	-
CLECBL™	-	-	-	-
Crystals (uncross-linked)	+	+	+	-

TABLE XII

Detergent Liquid Incubation Study - Dissolution Study - 200 fold Dilution 20 minutes at 52°C

Catalyst	Ciba #15	Ciba #16	Ciba #44	Tide
OA 1%, 16h	-/+	-/+	+	-
OA 0.5%, 16h	-	+	+	-
OA 0.1%, 16h	-	+	+	+
OA 0.2%, 16h	-/+	+	+	-
GA 0.5%, 1h	-	-/+	+	-
GA 0.9%, 1h	-	+	+	-
GA 0.7%, 1h	-	-	+	-
EP 1.0%,20min	+	+	+	+
GP 0.08%,20min	-	+	+	-
CLECBL™	-	-	-	-
Crystals (uncross-linked)	+	+	+	+

As demonstrated in the tables above, crosslinked enzyme crystals according to this invention are essentially insoluble in concentrated detergent and essentially soluble in diluted detergent under wash conditions.

Example 8 - Summary of Properties of Crosslinked Enzyme Crystals of This Invention

Table XIII below summarizes the overall stability/instability, activity and dissolution properties in Ciba detergent #15 of crosslinked subtilisin crystals prepared according to this invention using dialdehydes.

TABLE XIII

Cross-linker	Solubility in Ciba #15	Solubility on Dilution	Activity (t=0)	Stability at 52°C
Glyoxal	low	dissolve at 52°C	high	low
Succini-maldehyde	low	dissolve at 52°C; partially dissolve at 25°C	17-66% of Alcalase	ND
Glutaral-dehyde	very low	dissolve at 52°C; partially to fully dissolve at 25°C	1-100% of Alcalase	low 52°C moderate 40°C
Octane-dialdehyde	very low%	dissolve at 52°C; partially to fully dissolve at 25°C	30-66% of Alcalase	low 52°C moderate 40°C

As demonstrated in Table XIII above, the crosslinked enzyme crystals of the present invention are insoluble and, therefore, stable under storage conditions, while quickly releasing their activity under conditions of use. Advantageously, the crosslinked enzyme crystals of this invention exhibit activity similar to their soluble or uncrosslinked crystallized counterparts under conditions of use,

while displaying 5-6 fold improved stability, as well as favorable dissolution properties.

Example 9 - Effect of Change of Chemical Composition On Crosslinked Subtilisin Crystals

We crystallized subtilisin as described in Example 1 and crosslinked the resulting crystals as described in Example 2, using GA 1%/1 hour. When 100 μL (2.2 mg) of the resulting crosslinked subtilisin crystals was suspended in 1.5 mL of 33.3% of acetonitrile/phosphate buffer (0.3 M, pH 7.5), the crystals were completely dissolved after 45 minutes at 40°C.

Using similar conditions, suspending the crosslinked subtilisin crystals in 1.2 mL of 16.7% acetonitrile/buffer, the crystals were completely dissolved after 5 hours.

Activity (U)

time	in 100% buffer	ACN/16.7% Buffer	ACN/33.3% Buffer
0	27.3	27.3	27.3
1.5h	27.3	--	7.4*
3.3h	27.3	25.5	
7.0h	27.3	24.0**	

* crystals were completely dissolved at this time.
** crystals were not completely dissolved.

Assay: 0.2 mmol (75.8 mg) of TAME in 2.5 mL phosphate buffer was incubated with each crosslinked subtilisin crystal sample (equal to 0.044 mg enzyme crystals) suspension (or solution) at room temperature.

One unit hydrolyzed 1.0 µmole of TAME per min. from per mg crosslinked crystals.

The results above illustrate the trigger of addition of organic solvent to the environment of crosslinked protein crystals of this invention.

Example 10 - Wash Performance of Detergents Containing Crosslinked Subtilisin Crystals

We assessed the activity and storage stability of crosslinked enzyme crystals of this invention in liquid detergent, using a washing assay designed to test the ability of the detergent to remove stains from a fabric.

Washing Assay

Preparation of fabric

Cloth samples of the same size and weight were cut from the same bolt:

5 g of soiled test cloth and

5 g of cotton ballast with no soil (Ciba No. 1-3005).

Prior to washing the samples, we measured the light intensity (= lightness) remitted by the soiled fabric samples (as described below).

Preparation of detergent solution

The sample of liquid detergent to be tested was heated in a flask for two hours at 20°C. The sample was then homogenized by vigorous shaking and 0.8 g of the detergent was removed from the flask and added to 200 ml of tap water (20°C) in a metallic beaker. The aqueous detergent solution was stirred for 60 seconds.

Washing

A sample of soiled test cloth and a sample of unsoiled ballast were placed together into the beaker containing the aqueous detergent solution. The beaker was closed tightly and immediately inserted into a pre-heated (40°C) washing machine (Unitest, manufactured by Hereus, Switzerland). During the washing process, the beaker was rotated constantly in a water bath heated to 40°C. As a result, the contents of the beaker continuously warmed, up to a temperature of 40°C.

Exactly 20 minutes after the fabric was placed in the detergent solution, washing was stopped and the washed fabric was immediately removed from the detergent solution and rinsed for 30 seconds with cold tap water (13 - 15°C). The wet fabric was centrifuged and ironed to remove wrinkles and dried at the same time.

Measurement of washing performance

Each sample of the washed and dried fabric was examined for stain removal by remission measurements (lightness Y) between 460 and 700 nm using a Spectraflash 500 (Datacolor). A cut off filter was used to eliminate potential interference by contamination with UV-absorbing materials. The lightness value of each test cloth was measured 5x and an average calculated.

With increasing washing performance, the lightness of the fabric increases. Washing performance is thus defined as a difference in lightness, ΔY:

ΔY = Lightness of fabric after washing - Lightness of fabric before washing

Example 11 - Effect of Concentration of Crosslinked Subtilisin Crystals on Washing Performance of Detergents Containing Them

Washing performance of crosslinked enzyme crystals according to this invention was examined as a function of their concentration in the liquid detergent, using the materials described below.

Test fabric: EMPA (Eidgenössische Materialprüfungs und Forschungsanstalt, St. Gallen, Switzerland) #116 soiled with a combination of blood, milk and carbon black.

Liquid detergent: Ciba detergent #16.

Enzyme: - Crosslinked enzyme crystals; sample Altus IV (as described in Example 6)
- Uncrosslinked enzyme (Alcalase).

Concentration of enzyme in liquid detergent: enzyme concentrations were between 0.05 and 0.9 w% (dry matter weight). Table XIV provides further details.

TABLE XIV

Enzyme w%	Weight of enzyme suspension (g)		Dry matter weight of enzyme	Liquid Detergent Ciba #16
	Alcalase	Altus IV	g	g
0.05	0.106		0.0053	10
0.05		0.130	0.0056	10
0.1	0.207		0.0104	10
0.1		0.240	0.0104	10
0.3	0.599		0.0301	10
0.3		0.683	0.0297	10
0.5	0.492		0.0247	5
0.5		0.580	0.0252	5

Enzyme w%	Weight of enzyme suspension (g)		Dry matter weight of enzyme	Liquid Detergent Ciba #16
	Alcalase	Altus IV	g	g
0.9	0.886		0.0445	5
0.9		1.046	0.0455	5

Preparation of liquid detergent with enzyme

Specific aliquots of the suspension of enzyme crystals (see Table XIV) were added to a flask and centrifuged to separate the crystals from the liquid. The liquid was discarded and the crystals were suspended and homogenized in the liquid detergent (for quantities see Table XIV). The resulting preparations were used in the washing tests.

Washing tests to evaluate the performance of the enzyme detergent formulations were carried out as described in the assay above. The results of the study are depicted in Figure 2. The figure demonstrates that at enzyme concentrations ≥ 0.1 w%, the washing effect of Ciba liquid detergent #16 formulated with Altus IV exceeds that of the formulation with uncrosslinked Alcalase. The efficacy of both crosslinked and uncrosslinked enzymes was reduced at enzyme concentrations below 0.1 w%.

Example 12 - Storage Stability and Washing Performance of Detergents Containing Crosslinked Subtilisin Crystals

Detergents formulated with crosslinked and uncrosslinked enzymes were stored at a constant temperature, in order to examine enzyme stability in concentrated liquid detergent. The detergent

formulations (150 g each) were prepared by the same procedure as the samples in Example 10.

Liquid detergent: Ciba detergent #16.

Enzyme: - Crosslinked enzyme crystals: sample Altus IV (Example 6)

- Uncrosslinked enzyme (Alcalase)

Enzyme concentration: 0.3 w% (dry matter) in liquid detergent.

Storage temperature for stability studies:

All samples were stored at 30°C for between 0 and 7 days. After 7 days, the samples were divided after 7 days into two equal portions, in order to study stability at elevated temperature. One portion continued to be stored at 30°C, while the other was stored at 40°C.

Test fabric: Three different soiled fabrics were used. All of them were standard test materials available from EMPA:

- EMPA #112: cocoa soiled fabric
- EMPA #116: blood, milk and carbon black soiled fabric
- EMPA #111: blood soiled fabric.

Washing performance on cocoa soiled fabric

Washing performance of various enzyme formulated liquid detergents was studied with respect to removal of cocoa stains from a cocoa soiled test fabric, using the washing assay described above. Storage stability was determined by assessing washing performance periodically during the detergent storage time, thus monitoring the impact of storage temperature on enzyme performance in the liquid detergent. In this

assay, the effectiveness of the liquid detergent decreases as enzyme stability degrades. The results of this assay, shown in Figures 3 and 4, are discussed below.

Storage Stability at 30°C

As demonstrated in Figure 3, both Alcalase and Altus IV formulated detergents exhibited an improved performance after 2 days of storage (compared to initial values). However, as storage time increased, the performance of the Alcalase formulation decreased continuously over time, while the Altus IV formulated detergent exhibited no degradation, even after 28 days of storage.

Storage Stability at 40°C

As demonstrated in Figure 4, when the temperature was raised from 30 to 40°C, Alcalase formulated detergent lost activity within 2 days, while the Altus IV formulated detergent degraded slightly, while removing the cocoa soil from the test fabric significantly, even after 21 days of storage at 40°C.

Washing performance on fabric soiled by a combination of blood, milk and carbon black (EMPA #116 test fabric)

The experimental conditions and detergents were the same (except the stained fabric) as for washing of cocoa stains. The results of the washing tests are depicted in Figures 5 and 6.

Storage Stability at 30°C

Figure 5 clearly illustrates the decay of washing performance of the Alcalase formulated detergent after 2 days of storage at 30°C. However,

liquid detergent containing Altus IV enzyme maintained its original washing performance, even after 28 days of storage.

Storage Stability at 40°C

As demonstrated in Figure 6, when the storage temperature was raised from 30 to 40°C, Alcalase formulated detergent lost nearly all of its washing performance within 2 days. In contrast, detergent containing Altus IV retained its washing power for an additional 14 days.

Washing performance on fabric soiled with blood

Washing performance on blood stains was tested with enzyme containing detergents stored at 30°C. The detergent composition, washing conditions were the same as in washing of cocoa stains. The results of the washing test are illustrated in Figure 7.

The assays show that the washing effect on blood stain by Ciba #16 liquid detergent formulated with Alcalase was low in comparison to detergent without enzyme. On the other hand, the Altus IV formulation was more active in washing conditions and more stable in storage.

Storage Stability at 30°C

The washing effect of Alcalase formulated detergent decreased rapidly with storage time, whereas Altus IV formulated detergent retained almost completely its full capacity after 28 days of storage.

Example 13 - Solubility of Crosslinked Subtilisin Crystals at 30°C and 37°C

We studied the solubility of various subtilisin crystals, which had been crosslinked with glutaraldehyde (GA), octanedialdehyde (OA), neopentyl glycol diglycidyl ether (NP) followed by glutaraldehyde, or DENACOL EX-411 (411) followed by glutaraldehyde.

In 1.5 ml Eppendorf tubes, samples of uncrosslinked subtilisin crystals and crosslinked subtilisin crystal slurry, equal to 37.5 mg of enzyme, were microfuged at 5,000 rpm for 5 min and the supernatant liquid was removed. A 1.5 ml aliquot of PBS buffer (0.01 M phosphate, 0.0027 M potassium chloride, 0.137 M sodium chloride, pH 7.4) was added to each sample, bringing the concentration of subtilisin to 25 mg/ml. The samples were transferred to 2 ml glass vials with screw caps and magnetic stir bars then were incubated at 30°C or at 37°C. Samples were studied for dissolution by periodically removing 50 µl of the slurry, microfuging at 13,000 rpm for 5 mins, removing 20 µl of the aliquot and placing it in 980 µl of deionized water, then measuring UV absorbance at 280 nm.

The following samples were studied:

Crosslinker	Crosslinker Concentration	Crosslinking Time
GA	1.0%	1.5h
GA	0.25%	2h
GA	0.2%	2h
GA	0.15%	2h
NP/GA	0.1%/0.1%	5h/1.5h
411/GA	0.015%/0.035%	16h/1h
OA	0.2%	16h
OA	0.1%	16h
OA	0.05%	16h

The solubility profiles of the samples, shown in Figures 8 and 9, illustrate different rates of dissolution for the crosslinked crystals.

Example 14 - Reversible Crosslinkers - Disulfide Crosslinked Subtilisin Crystals

We prepared subtilisin crystals (30-40 µm average, 27 mg/ml in Na_2SO_4) as previously described for subtilisin crystallization.

We then crosslinked the crystals using one of the following crosslinkers:

1) Dimethyl 3, 3'-dithiobispropionimidate•HCl - (DTBP) (Pierce)
2) Dithiobis(succinimidylpropionate) - (DSP)(Pierce)
3) 3, 3'- Dithiobis (sulfosuccinimidylpropionate) - (DTSSP) (Pierce).

Crosslinking was carried out in 15 ml neoprene screw cap tubes by placing 740 µl of subtilisin crystal slurry (20 mg) in 9.26 ml of buffer (25 mM $NaCO_3$/50 mM $NaHCO_3$, pH 8.0). One crosslinker was

added to each tube as follows: A) 93 mg DTBP (30 mM) B) 100 mg DTSSP (16 mM) C) 120 mg DSP (30 mM).

The tubes were tumbled at ambient temperature (24-26°C) until all samples were determined to be insoluble in 32 mM NaOH (5 days) - 100 µl sample in 300 µl NaOH. Uncrosslinked samples were readily soluble in 32 mM NaOH at the same concentrations. Crosslinking was stopped by the addition of 1 ml of 1 M Tris, pH 7.5. The samples were centrifuged at 3,000 rpm for 5 minutes, the supernatant removed and replaced by 5 ml of 100 mM Tris, pH 7.5. Centrifugation at 3,000 rpm, for 5 min, followed by replacement of supernatant with 5 ml of 100 mM Tris (pH 7.5) was repeated 3x.

Example 15 - Dissolution of Disulfide Bond-Containing Crosslinked Subtilisin Crystals

A 200 mM solution of cysteine was prepared by dissolving 121 mg cysteine in 5 ml 100 mM Tris (pH 7.5). A 400 µl aliquot of the cysteine solution was added to 3 x 750 µl vials. A 400 µl aliquot of 100 mM Tris (pH 7.5) was added to another 3 x 750 µl vials. Each crosslinked sample (100 µl) was added to one vial containing cysteine and one vial without cysteine. All samples were incubated at 37°C and monitored for dissolution of crosslinked enzyme crystals (direct visual and microscopic observation).

After incubation for 3 hrs at 37°C, the DTBP sample appeared to be fully soluble in the presence of cysteine and insoluble in its absence. The DTSSP sample appeared to be nearly fully soluble in the presence of cysteine and insoluble in its absence. The DSP sample was barely soluble in the presence of cysteine and insoluble in its absence.

Example 16 - Crystallization of Candida Rugosa Lipase

A 5 kg aliquot of Candida rugosa lipase ("CRL") in powder form (Meito) was mixed with 5 kg celite and dissolved in 102 L distilled deionized water and the volume brought to 200 L with the deionized water. The suspension was mixed in an Air Drive Lightning Mixer for 2 hours at room temperature and then filtered through a 0.5 micron filter to remove celite. The mixture was then ultrafiltered and concentrated to 14 L (469 g) using a 3K hollow fiber filter membrane cartridge. Solid calcium acetate was added to a concentration of 5mM $Ca(CH_3COO)_2$. The pH was adjusted to pH 5.5 with concentrated acetic acid as necessary. A 7 litre aliquot was crystallized by either addition of 1.75 litres of 2-methyl-2,4-pentanediol ("MPD") or by addition of 3.5 litres of a 30% solution of PEG-8000. The resulting solution was mixed and crystallization allowed to proceed overnight at ambient temperature for about 17-20 hrs. The crystal yield was about 70%.

Recrystallization

The Candida rugosa lipase crystals were solubilized by the addition of 50 mM sodium phosphate (pH 5.2). Soluble protein concentration of the crystallization solution was adjusted to 20 mg/ml. MPD was added gradually with stirring over a 6-hour period, to a final concentration of 25%. The resulting solution was mixed and crystallization allowed to proceed at ambient temperature for 20 hours.

Example 17 - Crystallization of Candida Rugosa Lipase

Candida rugosa lipase crystals prepared as described in Example 16, prior to the solubilization

and recrystallization steps, were solubilized by the addition of 50 mM sodium acetate (pH 6.5). Soluble protein concentration of the crystallization solution was adjusted to 20 mg/ml. MPD was added gradually with stirring over a 6-hour period to a final concentration of 20%. The resulting solution was mixed and crystallization allowed to proceed at ambient temperature for 20 hours.

Example 18 - Crosslinking of Candida Rugosa Lipase Crystals

Candida rugosa lipase crystals, prepared as described in Example 16, were crosslinked by addition of untreated neat glutaraldehyde (Sigma) by adding 2 ml of 20% glutaraldehyde stepwise in a 40.5 ml volume over one hour to 8 ml of stirred lipase crystals (25 mg/ml), at ambient temperature. The final crosslinker concentration was 4.0%. Crosslinking was allowed to proceed over 24 hours. Crystals were recovered by low speed centrifugation and washed with 25% MPD in 50 mM sodium phosphate (pH 5.2).

Example 19 - Crosslinking of Candida Rugosa Lipase Crystals

Candida rugosa lipase crystals, prepared as described in Example 16, were crosslinked by addition of untreated neat glutaraldehyde by adding 2 ml of 20% glutaraldehyde gradually over a one hour period. Crystals were crosslinked and processed as described in Example 18.

Example 20 - Crosslinking of Candida Rugosa Lipase Crystals

Candida rugosa lipase crystals, prepared as described in Example 16, were crosslinked as described in in Example 19, except that the reaction was allowed to proceed for 24 hours. The crystals were then processed as described in Example 18.

Example 21 - Crosslinking of Candida Rugosa Lipase Crystals

Candida rugosa lipase crystals, prepared as described in Example 17, were crosslinked by addition of glutaraldehyde to a final concentration of 4.0%. Crosslinking was allowed to proceed for 3 hours. The crystals were processed as described in Example 18.

Example 22 - Crosslinking of Candida Rugosa Lipase Crystals

Candida rugosa lipase crystals, prepared as described in Example 17, were crosslinked in neat glutaraldehyde at a concentration of 6.5% for 1 hour. Crosslinking and processing were performed as described in Example 18.

Example 23 - Crosslinking of Candida Rugosa Lipase Crystals

Candida rugosa lipase crystals, prepared as described in Example 17, were crosslinked in neat glutaraldehyde at a concentration of 6.0% for 1 hour. Crosslinking and processing were performed as described in Example 18.

Example 24 - pH Controlled Solubility of Crosslinked Candida Rugosa Lipase Crystals

Solubility of various crosslinked Candida rugosa lipase crystals was studied following an increase in pH from 6.5 to 9.0. The crystals were incubated at 1 mg/ml in 50 mM sodium phosphate (pH 9) containing 25% MPD. Aliquots were removed after 3 hour and 24 hour incubation at 25°C with stirring. Activity and soluble protein concentration were measured as described in Example 25. The results are described in the table below.

Crosslinked Crystal Preparation	Time (hr) 3		24	
	Activity(U)	[Prot.] (mg/ml)	Activity(U)	[Prot.] (mg/ml)
Example 18	7.5	0.47	20	1
Example 19	10.8	0.60	11.3	0.63
Example 20	7.5	0.42	8.8	0.49

Example 25 - pH Solubility of Crosslinked Candida Rugosa Lipase Crystals

Solubility of various crosslinked Candida rugosa lipase crystals was studied following an increase in pH from 5.2 to 7.5. The crystals were incubated at 1 mg/ml in 50 mM sodium phosphate (pH 7.5) containing 25% MPD. Aliquots were removed after 3 hour and 24 hour incubation at 25°C with stirring. Insoluble material was removed by filtration (0.25 micron). Activity in solution was measured spectrophotometrically by monitoring the hydrolysis of para nitrophenyl acetate (Fluka) at 400 nm. Substrate concentration was 1 mM. The assay was performed at 25°C in a 1 ml volume of 50 mM sodium acetate (pH 6.5).

Soluble protein concentration was measured by absorbance at 280 mm. Results are presented in the table below.

Crosslinked Crystal Preparation	Time (hr) 3		24	
	Activity(U)	[Prot.] (mg/ml)	Activity(U)	[Prot.] (mg/ml)
Example 21	2.4	0.12	15	0.91
Example 22	10.0	0.63	15	1.0
Example 23	2.5	0.17	11	0.69

Example 26 - Crystallization of Human Serum Albumin

Human serum albumin ("HSA") was purchased from Sigma Chemical Company as a lyophilized powder. We added 10 grams of protein powder to a 75 ml stirred solution of 100 mM phosphate buffer pH 5.5 at 4°C. Final protein concentration was 120 mg/ml (determined from OD_{280}, extinction coefficient for serum albumin was assumed to equal 1). Saturated ammonium sulfate solution (767 g/l) prepared in deionized water was added to the protein solution to a final concentration of 50% saturation (350 g/1). The crystallization solution was "seeded" with 1 ml of albumin crystals (50 mg/ml) in 50% ammonium sulfate (pH 5.5). Seed crystals were prepared by washing a sample of crystals free of precipitate with a solution of 50% saturated ammonium sulfate in 100 mM phosphate buffer (pH 5.5). The seeded crystallization solution was incubated at 4°C overnight on a vigorously rotating platform. Crystal rods (20 µ) appeared in the solution overnight (16 hr).

Example 27 - Crosslinking of Human Serum Albumin Crystals

We crosslinked human serum albumin crystals, prepared as described in Example 18, at 4°C in a 10 ml stirred solution of crystals and mother liquor containing 50% saturated ammonium sulfate, as described above. The crystals, which were not washed prior to crosslinking, were crosslinked with glutaraldehyde as supplied by the manufacturer (Sigma). Glutaraldehyde ("GA") (20%) was added to the stirred crystallization solution in 4 equal volumes (62.5 µl) at 15 minute intervals to a final concentration of 0.5% (250 µl GA). The crystals were then incubated at 4°C. Aliquots were removed at incubation times 0, 30 min, 60 min and 4 hours incubation. Crosslinked albumin crystals were collected by low speed centrifugation and washed repeatedly with pH 7.5, 100 mM Tris HCl, 4°C. Washing was stopped when the crystals could be centrifuged at high speed without aggregation.

Example 28 - Crosslinking of Human Serum Albumin Crystals

We crosslinked human serum albumin crystals as described in Example 27 above, with one modification; glutaraldehyde (20%) was added to the crystallization solution in 4 equal volumes (131.3 µl) at 15 minute intervals to a final concentration of 1% (525 µl GA).

Example 29 - Solubility of Human Serum Albumin Crystals Crosslinked in 0.5% GA, time: 0 minutes incubation. Dissolution Induced by Elevated Temperature

Human serum albumin, crystallized as described in Example 26 and crosslinked for 0 minutes

in 0.5% glutaraldehyde, as described in Example 27, was assayed for solubility by incubating the crystals (20 mg/ml) with stirring, in phosphate buffered saline solution (pH 7.5) at room temperature ("RT") or at 37°C. Aliquots were removed for assay at times 0.5, 1, 4 and 24 hours. Insoluble material was removed from the solution by centrifugation and the soluble protein concentration was measured spectrophotometrically at 280 nm, as indicated in Table XV.

TABLE XV

Time (hr)	Soluble Protein (mg/ml)	
	RT	37°C
0.5	0.3	1.5
1.0	3	5
4.0	4	12.5
24.0	17	18.5

Example 30 - Solubility of Human Serum Albumin Crystals Crosslinked in 0.5% GA, time: 30 minutes incubation. Dissolution Induced by Elevated Temperature

Human serum albumin, crystallized as described in Example 26 and crosslinked in 0.5% glutaraldehyde, as described in Example 27, was assayed for solubility by incubating the crystals (20 mg/ml) in phosphate buffered saline solution (pH 7.5) at room temperature or at 37°C. Aliquots were removed for assay at times 0.5, 1, 4 and 24 hours. Insoluble material was removed from the solution by centrifugation and the soluble protein concentration was measured spectrophotometrically at 280 nm, as indicated in Table XVI.

TABLE XVI

Time (hr)	Soluble Protein (mg/ml)	
	RT	37°C
0.5	1.5	4
1.0	3	5.5
4.0	7	10
24.0	13.5	17.5

Example 31 - Solubility of Human Serum Albumin Crystals Crosslinked in 0.5% GA, time: 60 minutes incubation. Dissolution Induced by Elevated Temperature

Human serum albumin, crystallized as described in Example 26 and crosslinked with 0.5% glutaraldehyde, as described in Example 27, was assayed for solubility by incubating the crystals (20 mg/ml) in phosphate buffered saline solution (pH 7.5) at room temperature or at 37°C. Aliquots were removed for assay at times 0.5, 1, 4 and 24 hours. Insoluble material was removed from the solution by centrifugation and the soluble protein concentration was measured spectrophotometrically at 280 nm, as indicated in Table XVII.

TABLE XVII

Time (hr)	Soluble Protein (mg/ml)	
	RT	37°C
0.5	0	0.4
1.0	0	0.6
4.0	0	3
24.0	8	17

Example 32 - Solubility of Human Serum Albumin Crystals Crosslinked in 0.5% GA, time: 240 minutes incubation. Dissolution Induced by Elevated Temperature

Human serum albumin, crystallized as described in Example 26 and crosslinked with 0.5% glutaraldehyde, as described in Example 27, was assayed for solubility by incubating the crystals (20 mg/ml) in phosphate buffered saline solution (pH 7.5) at room temperature or at 37°C. Aliquots were removed for assay at times 0.5, 1, 4 and 24 hours. Insoluble material was removed from the solution by centrifugation and the soluble protein concentration was measured spectrophotometrically at 280 nm, as indicated in Table XVIII.

TABLE XVIII

Time (hr)	Soluble Protein (mg/ml)	
	RT	37°C
0.5	0	0
1.0	0.5	0
4.0	3.5	3
24.0	8.5	14.5

Example 33 - Solubility of Human Serum Albumin Crystals Crosslinked in 1.0% GA, time: 0 minutes incubation. Dissolution Induced by Elevated Temperature

Human serum albumin, crystallized as described in Example 26 and crosslinked as described in Example 27, was assayed for solubility by incubating the crystals (20 mg/ml) in phosphate buffered saline solution (pH 7.5) at room temperature or at 37°C. Aliquots were removed for assay at times 0.5, 1, 4 and 24 hours. Insoluble material was removed from the

solution by centrifugation and the soluble protein concentration was measured spectrophotometrically at 280 nm, as indicated in Table XIX.

TABLE XIX

Time (hr)	Soluble Protein (mg/ml)	
	RT	37°C
0.5	1	2
1.0	3	7
4.0	10.5	16
24.0	19	18.5

Example 34 - Solubility of Human Serum Albumin Crystals Crosslinked in 1.0% GA, time: 30 minutes incubation. Dissolution Induced by Elevated Temperature

Human serum albumin, crystallized as described in Example 26 and crosslinked as described in Example 27, was assayed for solubility by incubating the crystals (20 mg/ml) in phosphate buffered saline solution (pH 7.5) at room temperature or at 37°C. Aliquots were removed for assay at times 0.5, 1, 4 and 24 hours. Insoluble material was removed from the solution by centrifugation and the soluble protein concentration was measured spectrophotometrically at 280 nm, as indicated in Table XX.

TABLE XX

Time (hr)	Soluble Protein (mg/ml)	
	RT	37°C
0.5	0	0
1.0	0	2
4.0	4.5	7
24.0	8	13

Example 35 - Solubility of Human Serum Albumin Crystals Crosslinked in 1.0% GA, time: 60 minutes incubation. Dissolution Induced by Elevated Temperature

Human serum albumin, crystallized as described in Example 26 and crosslinked as described in Example 27, was assayed for solubility by incubating the crystals (20 mg/ml) in phosphate buffered saline solution (pH 7.5) at room temperature or at 37°C. Aliquots were removed for assay at times 0.5, 1, 4 and 24 hours. Insoluble material was removed from the solution by centrifugation and the soluble protein concentration was measured spectrophotometrically at 280 nm, as indicated in Table XXI.

TABLE XXI

Time (hr)	Soluble Protein (mg/ml)	
	RT	37°C
0.5	0	0.5
1.0	0	1.5
4.0	1	4
24.0	9	13.5

Example 35 - Solubility of Human Serum Albumin Crystals Crosslinked in 1.0% GA, time: 240 minutes incubation. Dissolution Induced by Elevated Temperature

Human serum albumin, crystallized as described in Example 26 and crosslinked as described in Example 27, was assayed for solubility by incubating the crystals (20 mg/ml) in phosphate buffered saline solution (pH 7.5) at room temperature or at 37°C. Aliquots were removed for assay at times 0.5, 1, 4 and 24 hours. Insoluble material was removed from the solution by centrifugation and the soluble protein concentration was measured spectrophotometrically at 280 nm, as indicated in Table XXII.

TABLE XXII

Time (hr)	Soluble Protein (mg/ml)	
	RT	37°C
0.5	0	0
1.0	0	0
4.0	0	2
24.0	6	10.3

Example 36 - Crystallization of Thermolysin

Thermolysin was purchased from Diawa (Japan) as a lyophilized powder. Fifteen grams of protein powder were added to a 100 ml stirred solution of 10 mM calcium acetate (pH 11) at ambient temperature. The pH was maintained at 11 by addition of 2 N NaOH, until the thermolysin was completely solubilized. The pH was then adjusted to pH 7.5 by addition of 2 N acetic acid. Crystallization was allowed to proceed overnight at 4°C. Final protein concentration was 40 mg/ml

(determined from OD_{280}, extinction coefficient for thermolysin was assumed to equal 1.8). Crystals were recovered by centrifugation and recrystallized to obtain a more uniform crystal size. Recrystallization was performed in a manner nearly identical to that described for the initial crystallization. Crystals (40 mg/ml protein) were dissolved by addition of base at room temperature. The pH of the crystallization solution was adjusted to 6.5 and crystallization was permitted to proceed at ambient temperature. Crystal rods (50 μ) appeared in the solution overnight (16 hr).

Example 37 - Crosslinking of Thermolysin Crystals

Thermolysin crystals, prepared as described in Example 36, were suspended (50 mg/ml) in a 50 mM solution of sodium acetate (pH 6.5). Crystals were crosslinked with glutaraldehyde as supplied by the manufacturer (Sigma). Ten milliliters of glutaraldehyde (10%) were added gradually over a 1 hour period with stirring to a 10 ml suspension of crystals. After all of the glutaraldehyde was added, the crystallization solution incubated at ambient temperature. Aliquots were removed at incubation times 0.5, 1 and 3 hr. Crosslinked crystals were collected by low speed centrifugation and washed exhaustively with pH 7.5 50 mM Tris HCl, containing 10 mM calcium acetate.

Example 38 - Solubility of Thermolysin Crystals Crosslinked for 0.5 hr. Dissolution Induced by Removal of Calcium Ions by EDTA

Thermolysin, crystallized as described in Example 36 and crosslinked for 0.5 hr as described in Example 37, was assayed for solubility by incubating

the crystals (1 mg/ml) with stirring, in 10 mM Tris HCl (pH 7.2) containing 1 mM EDTA (Sigma) 40°C. One ml aliquots were removed for assay at times 0.5, 3 and 24 hours. Insoluble crystals were removed from the solution by filtration. One ml of 500 mM calcium acetate (pH 7.2) was added to each aliquot. Soluble protein concentration was measured spectrophotometrically at 280 nm. Enzymatic activity was measured spectrophotometrically by monitoring the hydrolysis of a dipeptide substrate, FAGLA (Feder). Substrate concentration was 1.67 mM. One unit is defined as the amount of enzyme required to hydrolyze 1 µmole of substrate in one minute at pH 7.2, 40°C. The activity of soluble thermolysin was 27 U/mg protein. Data is presented in Table XXIII.

TABLE XXIII

Time (hr)	Soluble Protein (% of Max)	Activity (% of Max)
0.5	80	30
3.0	103	97
24.0	100	91

Example 39 - Solubility of Thermolysin Crystals Crosslinked for 1 hr. Dissolution Induced by Removal of Calcium Ions by EDTA

Thermolysin, crystallized as described in Example 36 and crosslinked for 1 hr as described in Example 37, was assayed for solubility by incubating the crystals (1 mg/ml) with stirring, in 10 mM TrisHCl (pH 7.2) containing 1 mM EDTA 40°C. One ml aliquots were removed for assay at times 0.5, 3 and 24 hours. Insoluble crystals were removed from the solution by

filtration. One ml of 500 mM calcium acetate (pH 7.2) was added to each aliquot. Soluble protein concentration was measured spectrophotometrically at 280 nm. Enzymatic activity was measured spectrophotometrically by monitoring the hydrolysis of a dipeptide substrate, FAGLA (Feder). Substrate concentration was 1.67 mM. One unit is defined as the amount of enzyme required to hydrolyze 1 µmole of substrate in one minute at pH 7.2, 40°C. The activity of soluble thermolysin was 27 U/mg protein. Data is presented in Table XXIV.

TABLE XXIV

Time (hr)	Soluble Protein (% of Max)	Activity (% of Max)
0.5	7	11
3.0	24	29
24.0	104	87

Example 40 - Solubility of Thermolysin Crystals Crosslinked for 3 hr. Dissolution Induced by Removal of Calcium Ions by EDTA

Thermolysin, crystallized as described in Example 36 and crosslinked for 3 hr as described in Example 37, was assayed for solubility by incubating the crystals (1 mg/ml) with stirring, in 10 mM TrisHCl (pH 7.2) containing 1 mM EDTA 40°C. One ml aliquots were removed for assay at times 0.5, 3 and 24 hours. Insoluble crystals were removed from the solution by filtration. One ml of 500 mM calcium acetate (pH 7.2) was added to each aliquot. Soluble protein concentration was measured spectrophotometrically at 280 nm. Enzymatic activity was measured

spectrophotometrically by monitoring the hydrolysis of a dipeptide substrate, FAGLA (Feder). Substrate concentration was 1.67 mM. One unit is defined as the amount of enzyme required to hydrolyze 1 µmole of substrate in one minute at pH 7.2, 40°C. The activity of soluble thermolysin was 27 U/mg protein. Data is presented in Table XXV.

TABLE XXV

Time (hr)	Soluble Protein (% of Max)	Activity (% of Max)
0.5	2	0
3.0	2	0
24.0	100	73

Example 41 - Solubility of Thermolysin Crystals Crosslinked for 3 hr. Dissolution Induced by Removal of calcium ions by dilution

Thermolysin crystals, prepared as described in Example 36 and crosslinked for 3 hr as described in Example 37, were washed free of calcium containing buffer and assayed for solubility by incubating the crystals (1 mg/ml) with stirring in deionized water. One ml aliquots were removed for assay at times 0.5, 3 and 24 hours. Insoluble crystals were removed from the solution by filtration. One ml of 500 mM calcium acetate (pH 7.2) was added to each aliquot. Soluble protein concentration was measured spectrophotometrically at 280 nm. Enzymatic activity was measured spectrophotometrically by monitoring the hydrolysis of a dipeptide substrate, FAGLA (Feder). Substrate concentration was 1.67 mM. One unit is defined as the amount of enzyme required to hydrolyze 1

μmole of substrate in one minute at (pH 7.2), 40°C. The activity of soluble thermolysin was 27 U/mg protein. Data is presented in Table XXVI.

TABLE XXVI

Time (hr)	Soluble Protein (% of Max)	Activity (% of Max)
0.5	0	0
3.0	0	7
24.0	111	81

Example 42 - Crystallization of Glucose Isomerase

Glucose isomerase ("GA") was supplied by Cultor (Finland) as a crystal slurry. The enzyme was recrystallized by solubilizing a 50 ml volume of the crystal slurry at 50°C with stirring for 15 minutes. The solution was clarified by filtration and allowed to cool slowly at room temperature. Fifty micron crystals appeared within 5 hours. Crystals were recovered by low speed centrifugation and washed with 166 mM magnesium sulfate.

Example 43 - Crosslinking of Glucose Isomerase Crystals

Five hundred milligrams of glucose isomerase crystals, prepared as described in Example 42, were suspended in a 50 ml solution of 166 mM magnesium sulfate. The crystals were crosslinked with glutaraldehyde as supplied by the manufacturer (Sigma). Five milliliters of glutaraldehyde (10%) were added gradually over a 1 hour period with stirring to the 50 ml suspension. After all of the glutaraldehyde was added, the crystallization solution incubated at

ambient temperature. Aliquots were removed at incubation times 1, 3 and 24 hr. Crosslinked crystals were collected by low speed centrifugation and washed exhaustively with 50 mM Tris HCl (pH 7.0).

Example 44 - Solubility of Glucose Isomerase Crystals Crosslinked for 1 hr. Dissolution Induced by Removal of Calcium Ions by Dilution at 50°C

Glucose isomerase crystals, prepared as described in Example 42 and crosslinked for 1 hr as described in Example 43, assayed for solubility by incubating the crystals (1 mg/ml) with stirring in deionized water. One ml aliquots were removed for assay at times 1, 3 and 24 hours. Soluble protein concentration was measured spectrophotometrically at 280nm (OD280) (extinction coefficient for GI was assumed to equal 1). Enzymatic activity was measured spectrophotometrically by monitoring the conversion of fructose to glucose.

Glucose concentration was quantitated spectrophotometrically using a coupled enzyme assay containing hexokinase and glucose-6-phosphate dehydrogenase. The dehydrogenase uses NADP as a cofactor and the amount of NADPH formed in the reaction is stoichiometric with the concentration of substrate (glucose). The assay was purchased as a kit from Boehringer Mannheim and was used according to the manufacturer's instructions. One unit is defined as the amount of enzyme required to convert 1 µmole fructose to glucose in one minute at pH 7.0, 60°C. The activity of soluble glucose isomerase was 51 U/mg protein. Data is presented in Table XXVII.

TABLE XXVII

Time (hr)	Soluble Protein (% of Max)	Activity (% of Max)
0.5	8	0
3.0	52	31
24.0	100	57

Example 45 - Solubility of Glucose Isomerase Crystals Crosslinked for 3 hr. Dissolution Induced by Removal of Calcium Ions by Dilution at 50°C

Glucose isomerase crystals, prepared as described in Example 42 and crosslinked for 3 hr as described in Example 43, were assayed for solubility by incubating the crystals (1 mg/ml) with stirring in deionized water. One ml aliquots were removed for assay at times 1, 3 and 24 hours. Soluble protein concentration was measured spectrophotometrically at 280nm (OD280) (extinction coefficient for GI was assumed to equal 1). Enzymatic activity was measured spectrophotometrically by monitoring the conversion of fructose to glucose.

Glucose concentration was quantitated spectrophotometrically using the coupled enzyme assay containing hexokinase and glucose-6-phosphate dehydrogenase, as described in Example 44. Data is presented in Table XXVIII.

TABLE XXVIII

Time (hr)	Soluble Protein (% of Max)	Activity (% of Max)
0.5	2	0
3.0	10	6.5
24.0	86	43

Example 46 - Solubility of Glucose Isomerase Crystals Crosslinked for 24 hr. Dissolution Induced by Removal of Calcium Ions by Dilution at 50°C

Glucose isomerase crystals, prepared as described in Example 42 and crosslinked for 1 hr as described in Example 43, were assayed for solubility by incubating the crystals (1 mg/ml) with stirring in deionized water. One ml aliquots were removed for assay at times 1, 3 and 24 hours. Soluble protein concentration was measured spectrophotometrically at 280nm (OD280) (extinction coefficient for glucose isomerase was assumed to equal 1). Enzymatic activity was measured spectrophotometrically by monitoring the conversion of fructose to glucose.

Glucose concentration was quantitated spectrophotometrically using the coupled enzyme assay containing hexokinase and glucose-6-phosphate dehydrogenase, as described in Example 44. Data is presented in Table XXIX.

TABLE XXIX

Time (hr)	Soluble Protein (% of Max)	Activity (% of Max)
0.5	2	0
3.0	24	5
24.0	83	61

Example 47 - Preparation of Tablets Containing Crosslinked Protein Crystals According to this Invention

Tablets containing crosslinked protein crystals according to this invention may be prepared as follows. A suspension of crosslinked protein crystals is placed in 0.1 M sodium acetate, 20 mM calcium chloride and buffer (pH 7) and dried at 35°C. The resulting dried material may be mixed with sorbitol 50:50 by weight and granulated with Eudragit NE 30D (a neutral copolymer based on ethyl- and methylacrylate) or Eudagit RL 30D (an ammonio-methacrylate copolymer). The granules are dried (for example, for 16 hours at 40°C) and compressed to round tablets of about 5 mm diameter and weight of about 125 mg. The content of crosslinked protein crystals in such solid preparations is about 45% by weight. If the above-described preparation is made without using sorbitol, the resulting tablets contain about 63% by weight crosslinked protein crystals.

When introduced into water or aqueous buffer (such as the above-described acetate buffer) all the tablets disintegrate in a matter of 10 minutes under mild shaking at room temperature) producing particles less than 100 μm in size, the majority in the range of 4-10 μm. Microscopic examination reveals polymer-free

singular protein crystals, as the predominant species. The slurry obtained by disrupting the tablets is assayed titrimetrically using hydrolysis of N(α)-p-tosyl L-arginine methyl ester (TAME) at 25°C (pH 8). Activity corresponding to between about 50% and 80% of activity of an equal amount of crosslinked protein crystals (counting the indicated weight of the crosslinked crystals, rather than of the whole tablets) results.

Example 48 - Crystallization of *Candida Rugosa* Lipase

Candida rugosa lipase was prepared as described in Example 16. After the addition of solid calcium acetate to 5 mM, however, the pH was adjusted to 4.8 instead of pH 5.5 with concentrated acetic acid as necessary. Next, a seven liter aliquot was crystallized by the addition of 1.4 liters of MPD. The resulting solution was mixed and crystallization was allowed to proceed for 72 hours at 4°C.

In the following examples, unless otherwise indicated, lipase crystals were prepared according to this example.

Example 49 - Crosslinking of Lipase Crystals

Lipase crystals were crosslinked using the following crosslinkers: dimethyl 3, 3'-dithiobispropionimidate·HCl (DTBP); dithiobis (succinimidylpropionate) (DSP); bismaleimidohexane (BMH); bis[Sulfosuccinimidyl]suberate (BS); 1,5-difluoro-2,4-dinitrobenzene (DFDNB); dimethylsuberimidate·2HCl (DMS); disuccinimidyl glutarate (DSG); disulfosuccinimidyl tartarate (Sulfo-DST); 1-ethyl-3-[3-dimethylaminoproplyl]carbodiimide

hydrochloride (EDC); ethylene glycolbis [sulfo-succinimidylsuccinate] (Sulfo-EGS); N-[γ-maleimido-butyryloxy]succinimide ester (GMBS); N-hydroxysulfo-succinimidyl-4-azidobenzoate (Sulfo-HSAB); sulfosuccinimidyl-6-[α-methyl-α-(2-pyridyldithio) toluamido]hexanoate (Sulfo-LC-SMPT); bis-[β-(4-azidosalicylamido) ethyl]disulfide (BASED); NHS-PEG-Vinylsulfone (NHS-PEG-VS); and glutaraldehyde (GA).

Dimethyl 3,3'-dithiobispropionimidate·HCl(DTBP) Crosslinking

A dimethyl 3,3'-dithiobispropionimidate·HCl (DTBP) solution was prepared by dissolving 27.9 mg of DTBP in 60 µl of water. Next, 40 µl of this solution was added to 21 mg of lipase crystals in 1.5 ml of 10 mM HEPES buffer, pH 8.5 and containing 10 mM calcium chloride and 20% MPD. The crosslinking reaction was carried out at ambient temperature for 24 hours with tumbling. After 24 hours, the slurry was centrifuged at 3000 rpm for 5 minutes and the supernatant was discarded. The pellet was then suspended in 10 mM HEPES buffer, pH 7.5 and containing 10 mM calcium chloride and 20% MPD. An additional amount (20 µl) of DTBP solution was added and crosslinking was continued for another 24 hours. The crosslinking reaction was terminated by washing off the excess reagent with 10 mM sodium acetate buffer, pH 4.8 and containing 10 mM calcium chloride and 20% MPD (five washes with 1 ml of buffer).

Dithiobis(succinimidylpropionate) (DSP) Crosslinking

A dithiobis (succinimidylpropionate) (DSP) solution was prepared by dissolving 36 mg of DSP in

60 µl of dimethyl sulfoxide (DMSO). Next, 40 µl of this solution was added to 21 mg of lipase crystals in 1.5 ml of 10 mM HEPES buffer, pH 8.5 and containing 10 mM calcium chloride and 20% MPD. The crosslinking reaction was carried out at ambient temperature for 24 hours with tumbling. After 24 hours, the slurry was centrifuged at 3000 rpm for 5 minutes and the supernatant was discarded. The pellet was then suspended in 10 mM HEPES buffer, pH 7.5 and containing 10 mM calcium chloride and 20% MPD. An additional amount (20 µl) of DSP solution was added and crosslinking was continued for another 24 hours. The crosslinking reaction was terminated by washing off excess reagent with 10 mM sodium acetate buffer, pH 4.8 and containing 10 mM calcium chloride and 20% MPD (five washes with 1 ml of buffer).

Bis Maleimidohexane (BMH) Crosslinking

A bis maleimidohexane (BMH) solution was prepared by dissolving 12 mg of BMH in 40 µl of dimethyl sulfoxide (DMSO). Next, 40 µl of this solution was added to 21 mg of lipase crystals in 1.5 ml of 10 mM HEPES buffer, pH 7.5 and containing 10 mM calcium chloride and 20% MPD. The crosslinking reaction was carried out at ambient temperature for 24 hours with tumbling. After 24 hours, the slurry was centrifuged at 3000 rpm for 5 minutes and the supernatant was discarded. The pellet was then suspended in 10 mM HEPES buffer, pH 7.5 containing and 10 mM calcium chloride and 20% MPD. An additional amount (20 µl) of BMH solution was added and crosslinking was continued for another 24 hours. The crosslinking reaction was terminated by washing off the excess reagent with 10 mM sodium acetate buffer, pH 4.8

and containing 10 mM calcium chloride and 20% MPD (five washes with 1 ml of buffer).

Bis [Sulfosuccinimidyl]suberate (BS) Crosslinking

A bis [sulfosuccinimidyl]suberate (BS) solution was prepared by dissolving 29 mg of BS in 50 µl of water. Next, 40 µl of this solution was added to 21 mg of lipase crystals in 1.5 ml of 10 mM HEPES buffer, pH 8.5 and containing 10 mM calcium chloride and 20% MPD. The crosslinking reaction was carried out at ambient temperature for 24 hours with tumbling. After 24 hours, the slurry was centrifuged at 3000 rpm for 5 minutes and the supernatant was discarded. The pellet was then suspended in 10 mM HEPES buffer, pH 7.5 and containing 10 mM calcium chloride and 20% MPD. An additional amount (20 µl) of BS solution was added and crosslinking was continued for another 24 hours. The crosslinking reaction was terminated by washing off the excess reagent with 10 mM sodium acetate buffer, pH 4.8 and containing 10 mM calcium chloride and 20% MPD (five washes with 1 ml of buffer).

1,5-Difluoro-2,4-dinitrobenzene (DFDNB) Crosslinking

A 1,5-Difluoro-2,4-dinitrobenzene (DFDNB) solution was prepared by dissolving 10 mg of DFDNB in 40 µl of acetone. Next, 40 µl of this solution was added to 21 mg of lipase crystals in 1.5 ml of 10 mM HEPES buffer, pH 8.5 and containing 10 mM calcium chloride and 20% MPD. The crosslinking reaction was carried out at ambient temperature for 24 hours with tumbling. After 24 hours, the slurry was centrifuged at 3000 rpm for 5 minutes and the supernatant was discarded. The pellet was then suspended in 10 mM HEPES buffer, pH 7.5 and containing 10 mM calcium

chloride and 20% MPD. An additional amount (20 µl) of DFDNB solution was added and crosslinking was continued for another 24 hours. The crosslinking reaction was terminated by washing off the excess reagent with 10 mM sodium acetate buffer, pH 4.8 and containing 10 mM calcium chloride and 20% MPD (five washes with 1 ml of buffer).

Dimethylsuberimidate·2HCl (DMS) Crosslinking

A dimethylsuberimidate·2HCl (DMS) solution was prepared by dissolving 33 mg of DMS in 40 µl of dimethyl sulfoxide (DMSO). Next, 40 µl of this solution was added to 21 mg of lipase crystals in 1.5 ml of 10 mM HEPES buffer, pH 8.5 and containing 10 mM calcium chloride and 20% MPD. The crosslinking reaction was carried out at ambient temperature for 24 hours with tumbling. After 24 hours, the slurry was centrifuged at 3000 rpm for 5 minutes and the supernatant was discarded. The pellet was then suspended in 10 mM HEPES buffer, pH 7.5 and containing 10 mM calcium chloride and 20% MPD. An additional amount (20 µl) of DMS solution was added and crosslinking was continued for another 24 hours. The crosslinking reaction was terminated by washing off the excess reagent with 10 mM sodium acetate buffer, pH 4.8 and containing 10 mM calcium chloride and 20% MPD (five washes with 1 ml of buffer).

Disuccinimidyl glutarate (DSG) Crosslinking

A disuccinimidyl glutarate (DSG) solution was prepared by dissolving 17 mg of DSG in 50 µl of dimethyl sulfoxide (DMSO). Next, 40 µl of this solution was added to 21 mg of lipase crystals in 1.5 ml of 10 mM HEPES buffer, pH 8.5 and containing 10 mM

calcium chloride and 20% MPD. The crosslinking reaction was carried out at ambient temperature for 24 hours with tumbling. After 24 hours, the slurry was centrifuged at 3000 rpm for 5 minutes and the supernatant was discarded. The pellet was then suspended in 10 mM HEPES buffer, pH 7.5 containing 10 mM calcium chloride and 20% MPD. An additional amount of DSG solution (20 µl) was added and crosslinking was continued for another 24 hours. The crosslinking reaction was terminated by washing off the excess reagent with 10 mM sodium acetate buffer, pH 4.8 and containing 10 mM calcium chloride and 20% MPD (five washes with 1 ml of buffer).

Disulfosuccinimidyl tartarate (Sulfo-DST) Crosslinking

A disulfosuccinimidyl tartarate (Sulfo-DST) solution was prepared by dissolving 27 mg of Sulfo-DST in 50 µl of water. Next, 40 µl of this solution was added to 21 mg of lipase crystals in 1.5 ml of 10 mM HEPES buffer, pH 8.5 and containing 10 mM calcium chloride and 20% MPD. The crosslinking reaction was carried out at ambient temperature for 24 hours with tumbling. After 24 hours, the slurry was centrifuged at 3000 rpm for 5 minutes and the supernatant was discarded. The pellet was then suspended in 10 mM HEPES buffer, pH 7.5 and containing 10 mM calcium chloride and 20% MPD. An additional amount (20 µl) of Sulfo-DST solution was added and crosslinking was continued for another 24 hours. The crosslinking reaction was terminated by washing off the excess reagent with 10 mM sodium acetate buffer, pH 4.8 and containing 10 mM calcium chloride and 20% MPD (five washes with 1 ml of buffer).

1-Ethyl-3-[3-Dimethylaminopropyl] carbodiimide hydrochloride (EDC) Crosslinking

A 1-Ethyl-3-[3-Dimethylaminopropyl] carbodiimide hydrochloride (EDC) solution was prepared by dissolving 10 mg of EDC in 1 ml of water. Next, 200 µl of this solution and 5 mg of solid Sulfo-NHS was added to 21 mg of lipase crystals in 1.5 ml of 10 mM HEPES buffer, pH 8.5 and containing 10 mM calcium chloride and 20% MPD. The crosslinking reaction was carried out at ambient temperature for 24 hours with tumbling. After 24 hours, the slurry was centrifuged at 3000 rpm for 5 minutes and the supernatant was discarded. The pellet was then suspended in 50 mM MES buffer, pH 6 and containing 10 mM calcium chloride and 20% MPD. An additional amount of an EDC + Sulfo-NHS solution (200 µl + 5 mg Sulfo-NHS) was added and crosslinking was continued for another 24 hours. The crosslinking reaction was terminated by washing off the excess reagent with 10 mM sodium acetate buffer, pH 4.8 and containing 10 mM calcium chloride and 20% MPD (five washes with 1 ml of buffer).

Ethylene glycolbis[sulfosuccinimidylsuccinate] (Sulfo-EGS) Crosslinking

An ethylene glycolbis [sulfosuccinimidyl succinate] (Sulfo-EGS) solution was prepared by dissolving 33 mg of Sulfo-EGS in 40 µl water. Next, 40 µl of this solution was added to 21 mg of lipase crystals in 1.5 ml of 10 mM HEPES buffer, pH 8.5 and containing 10 mM calcium chloride and 20% MPD. The crosslinking reaction was carried out at ambient temperature for 24 hours with tumbling. After 24 hours, the slurry was centrifuged at 3000 rpm for 5 minutes and the supernatant was discarded. The pellet

was then suspended in 10 mM HEPES buffer, pH 7.5 and containing 10 mM calcium chloride and 20% MPD. An additional amount (20 µl) of Sulfo-EGS solution was added and crosslinking was continued for another 24 hours. The crosslinking reaction was terminated by washing off the excess reagent with 10 mM sodium acetate buffer, pH 4.8 and containing 10 mM calcium chloride and 20% MPD (five washes with 1 ml of buffer).

N-[γ-maleimidobutyryloxy]succinimide ester (GMBS) Crosslinking

An N-[γ-maleimidobutyryloxy]succinimide ester (GMBS) solution was prepared by dissolving 23 mg of GMBS in 50 µl of dimethyl sulfoxide (DMSO). Next, 40 µl of this solution was added to 21 mg of lipase crystals in 1.5 ml of 10 mM HEPES buffer, pH 8.5 and containing 10 mM calcium chloride and 20% MPD. The crosslinking reaction was carried out at ambient temperature for 24 hours with tumbling. After 24 hours, the slurry was centrifuged at 3000 rpm for 5 minutes and the supernatant was discarded. The pellet was then suspended in 10 mM HEPES buffer, pH 7.5 and containing 10 mM calcium chloride and 20% MPD. An additional amount (20 µl) of GMBS solution was added and crosslinking was continued for another 24 hours. The crosslinking was terminated by washing off the excess reagent with 10 mM sodium acetate buffer, pH 4.8 and containing 10 mM calcium chloride and 20% MPD (five washes with 1 ml of buffer).

N-hydroxysulfosuccinimidyl-4-azidobenzoate (Sulfo-HSAB) Crosslinking

An N-hydroxysulfosuccinimidyl-4-azidobenzoate (Sulfo-HSAB) solution was prepared by dissolving 5 mg

of Sulfo-HSAB in 40 µl of water. Next, 40 µl of this solution was added to 21 mg of lipase crystals in 1.5 ml of 10 mM HEPES buffer, pH 8.5 and containing 10 mM calcium chloride and 20% MPD. The crosslinking reaction was carried out at ambient temperature for 24 hours with tumbling. After 24 hours, the slurry was centrifuged at 3000 rpm for 5 minutes and the supernatant was discarded. The pellet was then suspended in 10 mM HEPES buffer, pH 8.5 and containing 10 mM calcium chloride and 20% MPD. A second crosslinking reaction was carried out at ambient temperature for 10 minutes with shaking and using a 254 nm UV light. The UV lamp was kept 2.5 cm away from the sample. After 10 minutes, the slurry was centrifuged at 3000 rpm for 5 minutes and the supernatant was discarded. The crosslinking reaction was terminated by washing off the excess reagent with 10 mM sodium acetate buffer, pH 4.8 and containing 10 mM calcium chloride and 20% MPD (five washes with 1 ml of buffer).

Sulfosuccinimidyl-6-[α -methyl-α-(2-pyridyldithio) toluamido] hexanoate (Sulfo-LC-SMPT) Crosslinking

A sulfosuccinimidyl-6-[α-methyl-α-(2-pyridyldithio)toluamido] hexanoate (Sulfo-LC-SMPT) solution was prepared by dissolving 12 mg of Sulfo-LC-SMPT in 60 µl of water. Next, 40 µl of this solution was added to 21 mg of lipase crystals in 1.5 ml of 10 mM HEPES buffer, pH 8.5 and containing 10 mM calcium chloride and 20% MPD. The crosslinking reaction was carried out at ambient temperature for 24 hours with tumbling. After 24 hours, the slurry was centrifuged at 3000 rpm for 5 minutes and the supernatant was discarded. The pellet was then suspended in 10 mM HEPES buffer, pH 7.5 and containing 10 mM calcium

chloride and 20% MPD. An additional amount (20 µl) of Sulfo-LC-SMPT solution was added and crosslinking was continued for another 24 hours. The crosslinking reaction was terminated by washing off the excess reagent with 10 mM sodium acetate buffer, pH 4.8 and containing 10 mM calcium chloride and 20% MPD (five washes with 1 ml of buffer).

Bis-[β-(4-azidosalicylamido) ethyl] disulfide (BASED) Crosslinking

A bis [β-(4azidosalicylamido) ethyl]disulfide (BASED) solution was prepared by dissolving 3 mg of BASED in 40 µl of dimethyl sulfoxide (DMSO). Next, 40 µl of this solution was added to 21 mg of lipase crystals in 1.5 ml of 10 mM HEPES buffer, pH 8.5 and containing 10 mM calcium chloride and 20% MPD. The crosslinking reaction was carried out at ambient temperature for 30 minutes with shaking under a 365 nm UV light. The UV lamp was shown on the sample from 2.5 cm away. After 30 minutes, the slurry was centrifuged at 3000 rpm for 5 minutes and the supernatant was discarded. The crosslinking reaction was terminated by washing off the excess reagent with 10 mM sodium acetate buffer, pH 4.8 and containing 10 mM calcium chloride and 20% MPD (five washes with 1 ml of buffer).

NHS-PEG-Vinylsulfone (NHS-PEG-VS) Crosslinking

An NHS-PEG-Vinylsulfone (NHS-PEG-VS) solution was prepared by dissolving 20 mg of NHS-PEG-VS in 50 µl in water. Next, 50 µl of this solution was added to 34 mg of lipase crystals prepared as in Example 16 in 1.5 ml of 10 mM HEPES buffer, pH 8.5 and containing 10 mM calcium chloride and 20% MPD. The crosslinking

reaction was carried out at ambient temperature for 24 hours with tumbling. After 24 hours, the slurry was centrifuged at 3000 rpm for 5 minutes and the supernatant was discarded. The pellet was then suspended in 10 mM HEPES buffer, pH 7.5 and containing 10 mM calcium chloride and 20% MPD. An additional amount (25 µl) of GMBS solution was added and crosslinking was continued for another 24 hours. The crosslinking was terminated by washing off the excess reagent with 10 mM sodium acetate buffer, pH 4.8 and containing 10 mM calcium chloride and 20% MPD (five washes with 1 ml of buffer).

Glutaraldehyde (GA) Crosslinking

Candida rugosa lipase crystals were prepared as described in Example 16. The crosslinking reaction was initiated by the addition of untreated, neat glutaraldehyde (Sigma) to the crystal solution to achieve a final crosslinker concentration of 0.3% or 0.5%. The crosslinking reaction was then allowed to proceed for 1 hour. The crosslinked crystals were recovered by low speed centrifugation and washed with 10 mM sodium acetate buffer, pH 4.8 and containing 10 mM calcium acetate and 20% MPD.

Example 50 - pH Solubility of Crosslinked *Candida Rugosa* Lipase Crystals at 37°C

The solubility of *Candida rugosa* lipase crystals crosslinked as described in Example 49 was evaluated. Crystals crosslinked with the following crosslinkers were included dimethyl 3, 3'-dithiobispropionimidate·HCl (DTBP), dithiobis (succinimidylpropionate) (DSP), bismaleimidohexane

(BMH), bis[Sulfosuccinimidyl] suberate (BS), 1,5-difluoro-2,4-dinitrobenzene (DFDNB), dimethylsuberimidate·2HCl (DMS), disuccinimidyl glutarate (DSG), disulfosuccinimidyl tartarate (Sulfo-DST), 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), ethylene glycolbis [sulfo-succinimidylsuccinate] (Sulfo-EGS), N-[γ-maleimido-butyryloxy]succinimide ester (GMBS), N-hydroxysulfo-succinimidyl-4-azidobenzoate (Sulfo-HSAB), sulfosuccinimidyl-6-[α-methyl-α-(2-pyridyldithio) toluamido]hexanoate (Sulfo-LC-SMPT), bis-[β-(4-azidosalicylamido) ethyl]disulfide (BASED) and glutaraldehyde (GA).

Samples of uncrosslinked lipase crystals prepared as in Example 16 and crosslinked lipase crystal slurry equal to 2.8 mg of crystalline enzyme were dispensed into 1.5 ml Eppendorf tubes and microfuged at 3000 rpm for 5 minutes. The supernatant liquid was removed and the solubility of the resulting crystals was evaluated at pH 7.4 and pH 2.0.

For pH 7.4, a 200 µl aliquot of a 0.01 M phosphate buffer with 0.0027 M potassium chloride and 0.137 M sodium chloride at pH 7.4 (PBS) was added to each sample. Under these conditions the concentration of lipase was 14 mg/ml. Next, the samples were incubated at 37°C for 24 hours.

For the pH 2.0 dissolution measurement, the crosslinked crystal samples were initially equilibrated in a 10 mM glycine·HCl buffer. The equilibration buffer was prepared by mixing 80% of 10 mM glycine·HCl buffer with 10 mM calcium chloride at pH 2.0, and 20% of MPD, for a final pH of 4.8. Equilibration was carried out overnight at 25°C with tumbling. The equilibrated samples were then microfuged at 300 rpm

for 5 minutes and the supernatant liquids discarded. The samples were centrifuged at 3000 rpm for 5 minutes and the pellet was suspended in 200 µl of the Glycine·HCl buffer, pH 2.0. Under these conditions, the concentration of lipase was 14 mg/ml. Finally, the samples were incubated at 37°C for 5 hours.

The amount of soluble protein in the supernatant after the 5 hour incubation at pH 2.0 or the 24 hour incubation at pH 7.4 was measured using the Bio-Rad Micro-protein assay. After the incubation period, the samples were centrifuged at 14,000 rpm for 5 minutes. Next, the supernatant was filtered through 0.22 µm cellulose acetate filter (Sigma Chemical Co.). The soluble protein concentration was then measured by diluting 2 µl of the filtered supernatant into 798 µl of deionized water. Next, 200 µl of Bio-Rad Protein assay reagent was added to this sample and incubated at ambient temperature for 5 minutes. The absorbance was measured at 595 nm wavelength and compared to a protein standard curve of 0-20 µg bovine serum albumin from Pierce.

Different triggers alone or in combination determine the kinetics of protein release. The following samples were studied and the results are described in the Table XXX below.

Table XXX

Trigger→	Agitation (PBS)	Agitation and Acid pH 2.0	Agitation and Pronase
	Protein in filtrate in mg		
Crosslinker	(24 hours)	(5 hours)	(2 hours)
DTBP	0.82	0.73	0.81
DSP	0.74	0.84	0.68
BMH	0.86	1.74	0.53
DSG	0.94	0.79	0.68
SULFO-DST	1.13	0.85	0.75
DFDNB	0	0.01	0.06
BASED	0.89	0.99	0.63
GMBS	0.9	0.86	0.83
BS	0.02	0	0.03
SULFO-HSAB	0.24	0.22	0.22
SULFO-EGS	1.29	0.46	0.64
SULFO-SMPT	0.4	0.09	0.06
EDC	0.07	0.16	0.02
DMS	1.09	0.83	0.81
GA (0.3%)	0.98	0.97	0.13
GA (0.5%)	0.5	0.09	0.02
Soluble	2.8	2.8	2.8

Example 51 - Lipase Activity using olive oil as

The activity of various crosslinked enzyme crystals was assessed by measuring the hydrolysis of a substrate containing olive oil. During the reaction, the pH was held constant by titrating with 0.05 M NaOH. The reaction was followed with a pH-Stat (Titralab™) electrode from Radiometer, controlled by a VIT90 Video Titrator and a ABU91 Autoburette.

Procedure:

First, 20 ml of olive oil emulsion was added to the reaction vessel and the reaction mixture was equilibrated to 37°C with stirring. Next, the pH was adjusted to 7.7 using 0.05 M NaOH and an aliquot (34 mg) of crosslinked crystals was added. The pH of the mixture was maintained at 7.7 by titrating with NaOH. The volume of base consumption vs. time (ml/min) was recorded and plotted. The slope of the initial linear portion of the curve was used to determine the initial reaction rate.

a) Temperature of reaction: assay vessel was equilibrated and maintained in 37°C water bath during course of reaction.

b) Calculation: Initial rate = base consumption = ml/min (NaOH) X min.

c) Specific activity (μ moles/min/mg protein) = initial rate x 1000 x concentration of the titrant/the amount of enzyme (mg).

d) Blank: Without enzyme - Buffer used in the place of enzyme in the reaction mixture.

Reagents:

a) 3.0 M NaCl (solution A): 34.8 grams of NaCl was added to 150 ml of distilled water and stired. Final volume was made up to 200 ml.

b) 75 mM $CaCI_2 \cdot 2H_20$ (solution B): 220 mg of $CaCI_2 \cdot 2H_20$ was added to 150 ml of distilled water with stirring. Once the $CaCI_2 \cdot 2H_20$ dissolved, the volume was made up to 200 ml.

c) Mix: Solution A (40 ml) was added to solution B (20 ml) and H_2O (100 ml).

d) 0.5% Albumin: 500 mg of albumin/100 ml distilled water, was prepared by dissolving 1 gm of bovine serum albumin Fraction V (Sigma) in water with gentle stirring (avoid forming foam). After dissolving, the volume was made up to 200 ml.

e) An olive oil emulsion was prepared by first dissolving 16.5 gm of gum arabic (Sigma) in 130 ml of reagent grade water. Once the gum had dissolved, the volume was increased to 180 ml with distilled water and the solution was filtered through cotton. Next, 20 ml of olive oil (Sigma) was added and an emulsion was generated by mixing in a Quick Prep mixer for 3 minutes.

f) Substrate: 50 ml of olive oil emulsion was added to 40 ml of Mix (c) and 10 ml of 0.5% albumin.

Example 52 - Activity of Crosslinked *Candida Rugosa* Lipase Crystals

The activity of *Candida rugosa* lipase crystals crosslinked as described in Example 51 was assayed using olive oil as substrate (Table XXXI). Crystals crosslinked with the following crosslinkers were assayed: dithiobis (succinimidylpropionate) (DSP); bismaleimidohexane (BMH); NHS-PEG-Vinylsulfone (NHS-PEG-VS); disuccinimidyl glutarate (DSG); 1-ethyl-3-[3-dimethylarninopropyl] carbodiimide hydrochloride (EDC); sulfosuccinimidyl-6-[α-methyl-α-(2-pyridyldithio) toluamido]hexanoate (sulfo-LC-SMPT); and glutaraldehyde (GA).

Table XXXI

Crosslinked Crystal Preparation (34 mg)	Volume added μl	Specific Activity units/mg
DSP	40	921
BMH	40	1623
NHS-PEG-VS	50	948
DSG	20	933
DSG	40	687
DSG	80	419
EDC	50	1420
EDC	100	1271
EDC	150	686
sulfo-LC-SMPT	20	5756
sulfo-LC-SMPT	100	5624
GA	0.3%	614
GA	0.5%	322
SOLUBLE LIPASE		732

Table XXXI above demonstrates the effects of various crosslinkers and degrees of crosslinking on *Candida rugosa* lipase crystals. The amino/sulfo reactive heterobifunctional crosslinker sulfo-LC-SMPT dramatically enhanced the activity of lipase (greater than 7 fold) to an olive oil substrate when compared to the soluble enzyme. The amino reactive (DSP) or the sulfhydryl reactive (BMH) homobifunctional crosslinkers showed slightly enhancing and approximately 2-fold enhancement of activity, respectively, when compared to soluble enzyme.

Example 53 - Reversible Crosslinkers - Disulfide Crosslinked *Candida Rugosa* Lipase Crystals

Candida rugosa lipase crystals were prepared as described in Example 48. Samples containing crystals of 20-30 µM in average size and a total protein of 42 mg/ml were crosslinked using either of the reversible crosslinkers:

(1) Dimethyl 3,3'-dithiobispropionimidate·HCl (DTBP) (Pierce), or

(2) Dithiobis (succinimidylpropionate)(DSP) (Pierce),

(3) Bis[2-(sulfosuccinimidooxycarbonyloxy)-ethyl]sulfone (sulfo-BSOCOES) (Pierce).

The crosslinking reaction was carried out in duplicate 1.5 ml microcentrifuge tubes (USA/Scientific) by placing 250 µl of lipase crystal slurry (11.5 mg) into 500 µl of buffer containing 10 mM HEPES, 10 mM calcium acetate, 20% MPD at pH 8.5. Next, the crosslinking reaction was initiated by adding one crosslinker solution to each tube as follows:

A) 50 mM DTBP - 27.9 mg of DTBP was dissolved in 60 µl of water and 20 µl of this solution was added to one tube with the crystals; and

B) 14.8 mM DSP - 35.0 mg of DSP was dissolved in 120 µl of DMSO and then 10 µl of this solution was added to one tube with the crystals.

C) 7.5 mM Sulfo-BSOCOES - 7.2 mg of sulfo-BSOCOES was dissolved in 60 µl of water and then 20 µl of this solution was added to one tube with the crystals.

The tubes with DTBP and DSP were tumbled at ambient temperature (24-26°C) for approximately 2 days or until the sample was determined to be insoluble in 32 mM NaOH. The tube with sulfo-BSOCOES was tumbled at ambient temperature (24-26°C) for approximately 2 days. The solubility test consisted of adding 50 µl of sample to 150 µl of 32 mM NaOH. In this test, uncrosslinked samples were readily soluble in 32 mM NaOH at the same concentrations. The crosslinking reaction was terminated by centrifuging the sample at 3000 rpm for 5 minutes. Next, the supernatant was discarded and washed 3 times with 1 ml of 10 mM Tris·HCl buffer containing 10 mM calcium chloride and 20% MPD at pH 7.0.

Example 54 - Dissolution of Disulfide Bond - Containing Crosslinked Candida Rugosa Lipase Crystals

A 200 mM solution of cysteine was prepared by dissolving 242 mg of cysteine in 10 ml of 10 mM TRIS HCl buffer containing 10 mM calcium chloride and 20% MPD at pH 7. A 200 µl sample of crosslinked crystal slurry was centrifuged at 3000 rpm for 5 minutes and the supernatant was discarded. The pellet was suspended in 200 µl of cysteine containing TRIS buffer.

Another 200 µl of crosslinked sample was taken and centrifuged at 3000 rpm for 5 minutes and the supernatant was discarded. This pellet was then suspended in 200 µl of 10 mM Tris·HCl buffer at pH 7.0 without cysteine. All samples were incubated at 37°C for 1 hour and monitored by direct visual and microscopic observation for dissolution in 32 mM NaOH.

The sample exposed to DTBP was fully soluble in the presence of 200 mM cysteine and insoluble in its absence after incubation for 1 hour at 37°C. The DSP sample was slightly soluble after 1 hour in the presence of cysteine and insoluble in its absence.

Example 55 - Dissolution by Base Cleavable Crosslinked Candida Rugosa Lipase Crystals

A 200 µl sample of crosslinked crystal slurry was centrifuged at 3000 rpm for 5 minutes and the supernatant was discarded. The pellet was suspended in 200 µl of Tris buffer and 600 µl of 32 mM NaOH. Another 200 µl of crosslinked sample was taken and centrifuged at 3000 rpm for 5 minutes and the supernatant was discarded. This pellet was then suspended in 200 µl of 10 mM Tris·HCl buffer at pH 7.0. All samples were incubated at 37°C for 1 hour and monitored by direct visual and microscopic observation for dissolution in 32 mM NaOH.

The sample exposed to sulfo-BSOCOES was fully soluble in the presence of NaOH and insoluble in its absence.

While we have hereinbefore described a number of embodiments of this invention, it is apparent that our basic constructions can be altered to provide other embodiments which utilize the processes and

compositions of this invention. Therefore, it will be appreciated that the scope of this invention is to be defined by the claims appended hereto rather than by the specific embodiments which have been presented hereinbefore by way of example.

We Claim:

1. A crosslinked protein crystal, said protein crystal being capable of change from insoluble and stable form to soluble and active form upon a change in the environment surrounding said crystal, said change being selected from the group consisting of: change in temperature, change in pH, change in chemical composition, change from concentrate to dilute form, change in oxidation-reduction potential of the solution, change in the incident radiation, change in transition metal concentration, change in flouride concentration, change in free radical concentration, change in metal chelater concentration, change in shear force acting upon the crystal and combinations thereof.

2. The crosslinked protein crystal according to claim 1, wherein said change from concentrate to dilute form comprises a change in solute concentration.

3. The crosslinked protein crystal according to claim 2, wherein said change in solute concentration comprises an increase or decrease in salt concentration.

4. The crosslinked protein crystal according to claim 3, wherein said change in solute concentration comprises a decrease in salt concentration.

5. The crosslinked protein crystal according to claim 2, wherein said change in solute

concentration comprises an increase or decrease in water concentration.

6. The crosslinked protein crystal according to claim 5, wherein said change in solute concentration comprises an increase in water concentration.

7. The crosslinked protein crystal according to claim 2, wherein said change in solute concentration comprises an increase or decrease in organic solvent concentration.

8. The crosslinked protein crystal according to claim 2, wherein said change in solute concentration comprises a decrease in detergent concentration.

9. The crosslinked protein crystal according to claim 2, wherein said change in solute concentration comprises a decrease in protein concentration.

10. The crosslinked protein crystal according to claim 1, wherein said change from concentrate to dilute form comprises a change in concentration of all solutes from about 2-fold to about 10,000-fold.

11. The crosslinked protein crystal according to claim 10, wherein said change from concentrate to dilute form comprises a change in concentration of all solutes from about 2-fold to about 700-fold.

12. The crosslinked protein crystal according to claim 1, wherein said change in pH comprises a change from acidic pH to basic pH.

13. The crosslinked protein crystal according to claim 1, wherein said change in pH comprises a change from basic pH to acidic pH.

14. The crosslinked protein crystal according to claim 1, wherein said change in temperature comprises an increase or decrease in temperature.

15. The crosslinked protein crystal according to claim 14, wherein said change in temperature is an increase in temperature from a low temperature between about 0°C and about 20°C to a high temperature between about 25°C and about 70°C.

16. The crosslinked protein crystal according to claim 1, wherein said active form of said protein is a form which is active against macromolecular substrates.

17. A crosslinked protein crystal, said protein crystal having a half-life of activity under storage conditions which is greater than at least 2 times that of the soluble form of the protein that is crystallized to form said crystal that is crosslinked and activity similar to that of the soluble form of the protein under conditions of use.

18. A crosslinked protein crystal, said protein crystal being capable of releasing its protein activity at a controlled rate upon exposure to a change in the environment surrounding said crystal, said change being selected from the group consisting of change in pH, change in solute concentration, change in temperature, change in chemical composition, change in shear force acting upon the crystals and combinations thereof.

19. A crosslinked protein crystal, said protein crystal having about a 5 to 10 fold higher protein activity for any one of a macromolecular substrate, a biphasic substrate or a small molecule substrate, as compared with the soluble form of the protein that is crystallized to form the crystals that are crosslinked.

20. A crosslinked protein crystal, said protein crystal having about a 2 to 3 fold higher protein activity for any one of a macromolecular substrate, a biphasic substrate or a small molecule substrate, as compared with the soluble form of the protein that is crystallized to form the crystals that are crosslinked.

21. A crosslinked protein crystal, said protein crystal having about at least 1.2 times higher protein activity for a macromolecular substrate, a biphasic substrate or a small molecule substrate, as compared with the soluble form of the enzyme that is crystallized to form the crystals that are crosslinked.

22. A crosslinked lipase crystal, said lipase crystal having about a 5 to 10 fold higher enzyme activity for hydrolysis of a biphasic olive oil substrate, as compared with the soluble form of the lipase that is crystallized to form the crystals that are crosslinked.

23. The crosslinked lipase crystal according to claim 22, said lipase crystal having about a 2 to 3 fold higher enzyme activity for hydrolysis of a biphasic olive oil substrate, as compared with the soluble form of the lipase that is crystallized to form the crystals that are crosslinked.

24. The crosslinked lipase crystal according to claim 23, said lipase crystal having about at least 2 times higher protein activity for hydrolysis of a biphasic olive oil substrate, as compared with the soluble form of the lipase that is crystallized to form the crystals that are crosslinked.

25. The crosslinked lipase crystal according to any one of claims 22, 23 or 24, wherein said protein is crosslinked by sulfosuccinimidyl-6-[α-methyl-α-(2-pyridyldithio) toluamido] hexanoate (Sulfo-LC-SMPT).

26. The crosslinked lipase crystal according to any one of claims 22, 23 or 24, wherein said protein is crosslinked by 1-ethyl-3-[3-dimethylaminoproplyl]carbodiimide hydrochloride (EDC).

27. The crosslinked lipase crystal according to any one of claims 22, 23 or 24, wherein said lipase

is crosslinked by sulfosuccinimidyl-6-[α-methyl-α-(2-pyridyldithio) toluamido] hexanoate (Sulfo-LC-SMPT).

28. The crosslinked lipase crystal according to any one of claims 22, 23 or 24, wherein said lipase is crosslinked by a crosslinker selected from the group consisting of 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), dithiobis (succinimidylpropionate) (DSP), bismaleimidohexane (BMH), NHS-PEG-Vinylsulfone (NHS-PEG-VS) and disuccinimidyl glutarate (DSG).

29. The crosslinked protein crystal according to any one of claims 1, 17, 18, 19, 20 or 21, wherein said protein is crosslinked by a homobifunctional crosslinker.

30. The crosslinked protein crystal according to any one of claims 1, 17, 18, 19, 20, or 21, wherein said protein is crosslinked by a heterobifunctional crosslinker.

31. The crosslinked protein crystal according to claim 18, wherein said controlled rate of releasing protein activity is determined by a factor selected from the group consisting of: the degree of crosslinking of said crosslinked protein crystal, the length of time of exposure of protein crystal to the crosslinker, the amino acids residues involved in the crosslinks, whether the crosslinker is homobifunctional or heterobifunctional, the rate of addition of the crosslinking agent to said protein crystal, the nature of the crosslinker, the chain length of the crosslinker, the surface area of said crosslinked

protein crystal, the size of said crosslinked protein crystal, the shape of said crosslinked protein crystal and combinations thereof.

32. The crosslinked protein crystal according to claim 18, wherein said crystal has a protein activity release rate of between about 0.1% per day and about 100% per day.

33. The crosslinked protein crystal according to claim 18, wherein said crystal has a protein activity release rate between about 0.01% per hour and about 100% per hour.

34. The crosslinked protein crystal according to claim 18, wherein said crystal has a protein activity release rate between about 1% per minute and about 50% per minute.

35. The crosslinked protein crystal according to any one of claims 1, 17, 18, 19, 20 or 21, said protein crystal being substantially insoluble and stable in a composition under storage conditions and substantially soluble and active under nditions of use of said composition.

36. The crosslinked protein crystal according to claim 35, wherein said composition is selected from the group consisting of cleaning agents, detergents, personal care compositions, cosmetics, pharmaceuticals, veterinary compounds, vaccines, foods, feeds, diagnostics and formulations for decontamination.

37. The crosslinked protein crystal according to claim 36, wherein said detergent is selected from the group consisting of powdered detergents, liquid detergents, bleaches, household cleaners, hard surface cleaners, industrial cleaners, carpet shampoos and upholstery shampoos.

38. The crosslinked protein crystal according to claim 36, wherein said cosmetic is selected from the group consisting of creams, emulsions, lotions, foams, washes, gels, compacts, mousses, sunscreens, slurries, powders, sprays, foams, pastes, ointments, salves, balms, shampoos, sunscreens and drops.

39. The crosslinked protein crystal according to any one of claims 1, 17, 18, 19, 20 or 21, wherein said protein is an enzyme.

40. The crosslinked protein crystal according to claim 39, wherein said enzyme is selected from the group consisting of hydrolases, isomerases, lyases, ligases, transferases and oxidoreductases.

41. The crosslinked protein crystal according to claim 40, wherein said enzyme is selected from the group consisting of proteases, amylases, cellulases, lipases and oxidases.

42. The crosslinked protein crystal according to any one of claims 1, 17, 18, 19, 20 or 21, wherein said protein is selected from the group consisting of therapeutic proteins, cleaning agent proteins, personal care proteins, veterinary proteins,

food proteins, feed proteins, diagnostic proteins and decontamination proteins.

43. The crosslinked protein crystal according to any one of claims 1, 17, 18, 19, 20 or 21, wherein said protein is selected from the group consisting of hormones, antibodies, inhibitors, growth factors, trophic factors, cytokines, lymphokines, toxoids, growth hormones, nerve growth hormones, bone morphogenic proteins, toxoids, vitamins and nutrients.

44. The crosslinked protein crystal according to any one of claims 1, 17, 18, 19, 20 or 21, wherein said protein is selected from the group consisting of insulin, amylin, erythropoietin, Factor VIII, TPA, dornase-α, α-1-antitripsin, urease, fertility hormones, FSH, LSH, postridical hormones, tetanus toxoid and diptheria toxoid.

45. The crosslinked protein crystal according to any one of claims 1, 17, 18, 19, 20 or 21, said crystal having a longest dimension of between about 0.01 μm and about 500 μm.

46. The crosslinked protein crystal according to any one of claims 1, 17, 18, 19, 20 or 21, said crystal having a longest dimension of between about 0.1 μm and about 50 μm.

47. The crosslinked protein crystal according to any one of claims 1, 17, 18, 19, 20 or 21, said crystal having a shape selected from the group consisting of: spheres, needles, rods, plates, rhomboids, cubes, bipryamids and prisms.

48. A composition comprising a crosslinked protein crystal according to any one of claims 1, 17, 18, 19, 20, 21, 22, 23, or 24 said composition being selected from the group consisting of cleaning agents, detergents, personal care compositions, cosmetics, pharmaceuticals, veterinary compounds, vaccines, foods, feeds, diagnostics and formulations for decontamination.

49. The composition according to claim 48, wherein said detergent is selected from the group consisting of powdered detergents, liquid detergents, bleaches, household cleaners, hard surface cleaners, industrial cleaners, carpet shampoos and upholstery shampoos.

50. The composition according to claim 48, wherein said cosmetic is selected from the group consisting of creams, emulsions, lotions, foams, washes, gels, compacts, sunscreens, slurries, powders, sprays, foams, pastes, ointments, salves, balms, shampoos, sunscreens and drops.

51. A protein delivery system, said system comprising crosslinked protein crystals according to any one of claims 1, 17, 18, 19, 20, 21, 22, 23, or 24.

52. The protein delivery system according to claim 51, wherein said protein is selected from the group consisting of: detergent enzymes, cosmetic proteins, pharmaceutical proteins, agricultural proteins, vaccine proteins and decontamination proteins.

53. The protein delivery system according to claim 52, said protein delivery system being a microparticulate protein delivery system.

54. The protein delivery system according to claim 53, wherein said microparticulate protein delivery system comprises crosslinked protein crystals having a longest dimension between about 0.01 μm and about 500 μm.

55. The protein delivery system according to claim 54, wherein said microparticulate protein delivery system comprises crosslinked protein crystals having a longest dimension of between about 0.1 μm and about 50 μm.

56. The protein delivery system according to claim 53, wherein said microparticulate protein delivery system comprises crosslinked protein crystals having a shape selected from the group consisting of: spheres, needles, rods, plates, rhomboids, cubes, bipryamids and prisms.

57. A detergent formulation comprising a crosslinked protein crystal according to any one of claims 1, 17, 18, 19, 20 or 21.

58. A controlled release formulation comprising a crosslinked protein crystal according to any one of claims 1, 17, 18, 19, 20 or 21.

59. A pharmaceutical controlled release formulation comprising a crosslinked protein crystal according to any one of claims 1, 17, 18, 19, 20 or 21.

60. A pharmaceutical controlled release formulation comprising a crosslinked protein crystal, said crystal being substantially insoluble under storage conditions and capable of releasing its protein activity in vivo at a controlled rate.

61. The pharmaceutical controlled release formulation according to claim 59, said pharmaceutical being capable of administration by parenteral or non-parenteral routes.

62. The pharmaceutical controlled release formulation according to claim 61, said pharmaceutical being capable of administration by oral, pulmonary, nasal, aural, anal, dermal, ocular, intravenous, intramuscular, intraarterial, intraperitoneal, mucosal, sublingual, subcutaneous or intracranial route.

63. The pharmaceutical controlled release formulation according to claim 59, wherein said pharmaceutical is capable of administration by oral route and said crosslinked protein crystal is substantially insoluble under gastric pH conditions and substantially soluble under small intestine pH conditions.

64. A vaccine comprising a crosslinked protein crystal according to any one of claims 1, 17, 18, 19, 20 or 21.

65. A formulation comprising a crosslinked protein crystal according to any one of claims 1, 17, 18, 19, 20, 21, 22, 23, or 24 said formulation being selected from the group consisting of tablets,

liposomes, granules, spheres, microspheres, microparticles and capsules.

66. A method for producing crosslinked protein crystals comprising the step of reacting protein crystals with a first crosslinking agent, or a first crosslinking agent and at least a second crosslinking agent, under conditions sufficient to induce crosslinking of said crystals to the extent that the resulting crosslinked crystals are characterized by the ability to change from insoluble and stable form to soluble and active form upon a change in their environment, said change being selected from the group consisting of change in temperature, change in pH, change in chemical composition, change from concentrate to dilute form, change in oxidation-reduction potential of the solution, change in the incident radiation, change in transition metal concentration, change in flouride concentration, change in free radical concentration, change in metal chelater concentration, change in shear force acting upon the crystals and combinations thereof.

67. A method for producing crosslinked protein crystals comprising the step of reacting protein crystals with a first crosslinking agent, or a first crosslinking agent and at least a second crosslinking agent, under conditions sufficient to induce crosslinking of said crystals to the extent that the resulting crosslinked crystals are characterized by a half-life of activity under storage conditions which is greater than at least 2 times that of the soluble form of the protein that is crystallized to form said crystals that are crosslinked and activity similar to

that of the soluble form of the protein under conditions of use.

68. A method for producing crosslinked protein crystals comprising the step of reacting protein crystals with a first crosslinking agent, or a first crosslinking agent and at least a second crosslinking agent, under conditions sufficient to induce crosslinking of said crystals to the extent that the resulting crosslinked crystals are characterized by being capable of releasing their protein activity at a controlled rate upon exposure to a change in their environment, said change being selected from the group consisting of change in pH, change in soluble concentration, change in temperature, change in chemical composition, change in oxidation-reduction potential of the solution, change in the incident radiation, change in transition metal concentration, change in flouride concentration, change in free radical concentration, change in metal chelater concentration, change in shear force acting upon the crystals and combinations thereof.

69. The method for producing crosslinked protein crystals according to any one of claims 66, 67 or 68, comprising the step of reacting said protein crystals with said first crosslinking agent and said at least a second crosslinking agent at the same time or in sequence.

70. The method for producing crosslinked protein crystals according to any one of claims 66, 67 or 68, wherein, prior to reacting protein crystals with

said crosslinking agent, said method further comprises the step of crystallizing said protein.

71. The method for producing crosslinked protein crystals according to any one of claims 66, 67 or 68, wherein the conditions sufficient to induce crosslinking are dependent upon a factor selected from the group consisting of: the degree of crosslinking of said crosslinked protein crystals, the length of time of exposure of protein crystals to the crosslinking agent, the rate of addition of the crosslinking agent to said protein crystal, the nature of the crosslinker, the chain length of the crosslinker, the surface area of said crosslinked protein crystals, the size of said crosslinked protein crystals, the shape of said crosslinked protein crystals and combinations thereof.

72. The method for producing crosslinked protein crystals according to claim 68, wherein said controlled rate of releasing protein activity is determined by a factor selected from the group consisting of: the degree of crosslinking of said crosslinked protein crystals, the length of time of exposure of protein crystals to the crosslinking agent, the rate of addition of the crosslinking agent to said protein crystals, the nature of the crosslinker, the chain length of the crosslinker, the surface area of said crosslinked protein crystals, the size of said crosslinked protein crystals, the shape of said crosslinked protein crystals and combinations thereof.

73. The method for producing crosslinked protein crystals according to any one of claims 66, 67

or 68, wherein said crosslinking agent is a multifunctional crosslinking agent.

74. The method for producing crosslinked protein crystals according to claim 73, wherein said crosslinking agent is a bifunctional crosslinking agent.

75. The method for producing crosslinked protein crystals according to claim 73, wherein said crosslinking agent is selected from the group consisting of: glutaraldehyde, succinaldehyde, octanedialdehyde and glyoxal.

76. The method for producing crosslinked protein crystals according to claim 73, wherein said crosslinking agent is selected from the group consisting of: halo-triazines, halo-pyrimidines, anhydrides of aliphatic or aromatic mono- or di-carboxylic acids, halides of aliphatic or aromatic mono- or di-carboxylic acids, N-methylol compounds, di-isocyanates, di-isothiocyanates and aziridines.

77. The method for producing crosslinked protein crystals according to any one of claims 66, 67 or 68, wherein said crosslinking agent is an epoxide.

78. The method for producing crosslinked protein crystals according to claim 77, wherein said epoxide is selected from the group consisting of: neopentyl glycol diglycidyl ether, ethylene glycol diglycidyl ether, di-epoxides, tri-epoxides and tetra-epoxides.

79. The method for producing crosslinked protein crystals according to any one of claims 66, 67 or 68, wherein said crosslinking agent is 0.0076% to 0.5% glutaraldehyde and wherein the conditions sufficient to induce crosslinking include reacting protein crystals with a crosslinking agent for a period of time between about 3 minutes and about 120 minutes.

80. The method for producing crosslinked protein crystals according to claim 79, wherein said crosslinking agent is 0.005% glutaraldehyde and wherein the conditions sufficient to induce crosslinking include reacting protein crystals with a crosslinking agent for a period of time between about 10 minutes and about 30 minutes.

81. The method for producing crosslinked protein crystals according to claim 79 wherein, prior to reaction with said protein crystals, said glutaraldehyde is pretreated by incubation at 60°C with a buffer for 1 hour.

82. The method for producing crosslinked protein crystals according to any one of claims 66, 67 or 68, wherein said crosslinking agent is 0.01% to 1% glyoxal and wherein the conditions sufficient to induce crosslinking include reacting protein crystals with a crosslinking agent for a period of time between about 30 minutes and about 60 minutes.

83. The method for producing crosslinked protein crystals according to any one of claims 66, 67 or 68, wherein said crosslinking agent is 0.05% to 1% octanedialdehyde and wherein the conditions sufficient

to induce crosslinking include reacting protein crystals with a crosslinking agent for a period of time between about 30 minutes and about 16 hours.

84. The method for producing crosslinked protein crystals according to claim 83, wherein said crosslinking agent is 1% octanedialdehyde and wherein the conditions sufficient to induce crosslinking include reacting protein crystals with a crosslinking agent for a period of time between about 1 hour and about 3 hours.

85. The method for producing crosslinked protein crystals according to any one of claims 66, 67 or 68, wherein said crosslinking agent is 1% succinaldehyde and wherein the conditions sufficient to induce crosslinking include reacting protein crystals with a crosslinking agent for a period of time between about 30 minutes and about 3 hours.

86. The method for producing crosslinked protein crystals according to any one of claims 66, 67 or 68, wherein said first crosslinking agent is 0.01% to 4% epoxide and said second crosslinking agent is 0.1% to 0.2% glutaraldehyde and wherein the conditions sufficient to induce crosslinking include reacting said protein crystals with said first crosslinking agent for a period of time between about 1 hour and about 72 hours and reacting said protein crystals with said second crosslinking agent for a period of time between about 1 hour and about 5 hours.

87. The method for producing crosslinked protein crystals according to claim 86, wherein said

first crosslinking agent is 0.01% epoxide and said second crosslinking agent is 0.1% glutaraldehyde and wherein the conditions sufficient to induce crosslinking include reacting said protein crystals with said first crosslinking agent for about 5 hours and reacting said protein crystals with said second crosslinking agent for about 1.5 hours.

88. The method for producing crosslinked protein crystals according to any one of claims 66, 67 or 68, wherein said protein is an enzyme.

89. The method for producing crosslinked protein crystals according to any one of claims 66, 67 or 68, wherein said crosslinking agent is a reversible crosslinking agent.

90. The method for producing crosslinked protein crystals according to claim 89, wherein said reversible crosslinking agent is a disulfide crosslinking agent.

91. The method for producing crosslinked protein crystals according to claim 90, wherein said disulfide crosslinking agent is a homobifunctional crosslinking agent or a heterobifunctional crosslinking agent.

92. The method for producing crosslinked protein crystals according to any one of claims 66, 67 or 68, wherein said protein is an enzyme.

93. The method for producing crosslinked protein crystals according to claim 92, wherein said

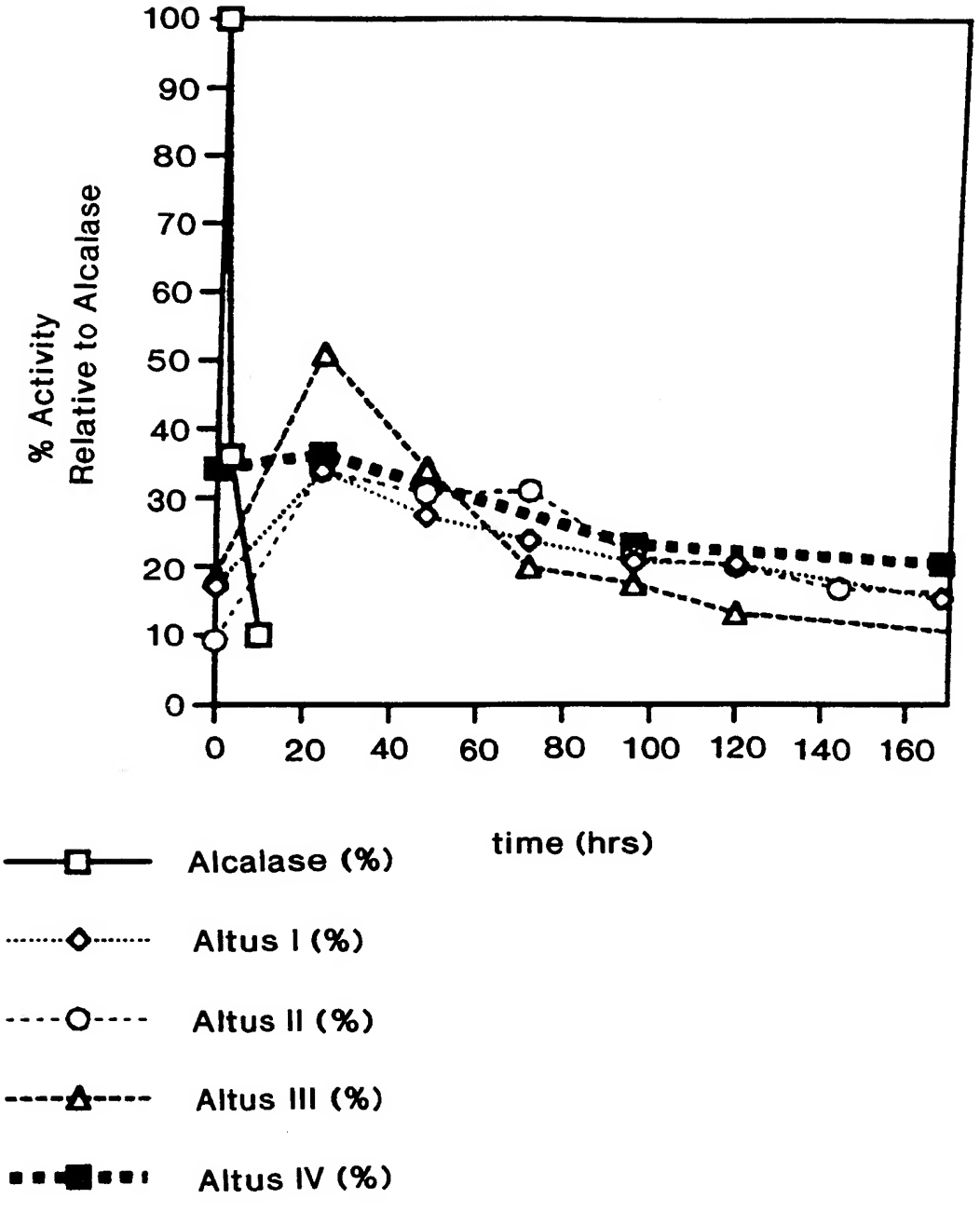
enzyme is selected from the group consisting of hydrolases, isomerases, lyases, ligases, transferases and oxidoreductases.

94. The method for producing crosslinked protein crystals according to claim 93, wherein said enzyme is from the group consisting of proteases, amylases, cellulases, lipases and oxidases.

95. The method for producing crosslinked protein crystals according to any one of claims 66, 67 or 68, wherein said protein is selected from the group consisting of therapeutic proteins, cleaning agent proteins, personal care proteins, veterinary proteins, food proteins, feed proteins, diagnostic proteins and decontamination proteins.

96. The method for producing crosslinked protein crystals according to claim 95, wherein said protein is selected from the group consisting of hormones, antibodies, inhibitors, growth factors, trophic factors, cytokines, lymphokines, toxoids, growth hormones, nerve growth hormones, bone morphogenic proteins, toxoids, vitamins and nutrients.

FIG. 1


100
90
80
70
60
50
40
30
20
10
0
% Activity
Relative to Alcalase
0
20
40
60
80
100
120
140
160
time (hrs)
Alcalase (%)
Altus I (%)
Altus II (%)
Altus III (%)
Altus IV (%)

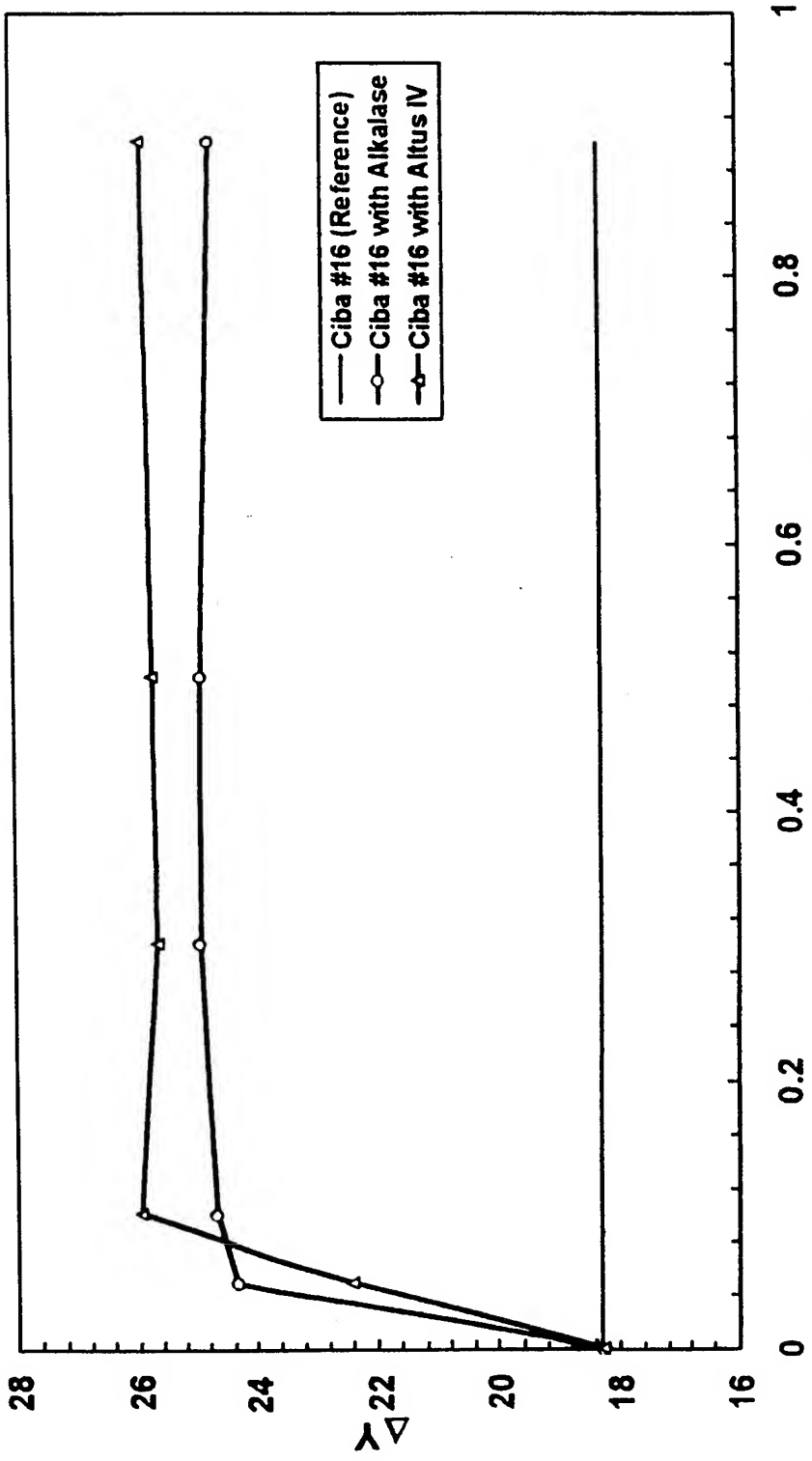

Ciba #16 (Reference)
Ciba #16 with Alkalase
Ciba #16 with Altus IV
Enzyme concentration (%w)
0
0.2
0.4
0.6
0.8
1
16
18
20
22
24
26
28
ΔY
Δ Y = Lightness after washing - Lightness before washing

FIG. 2

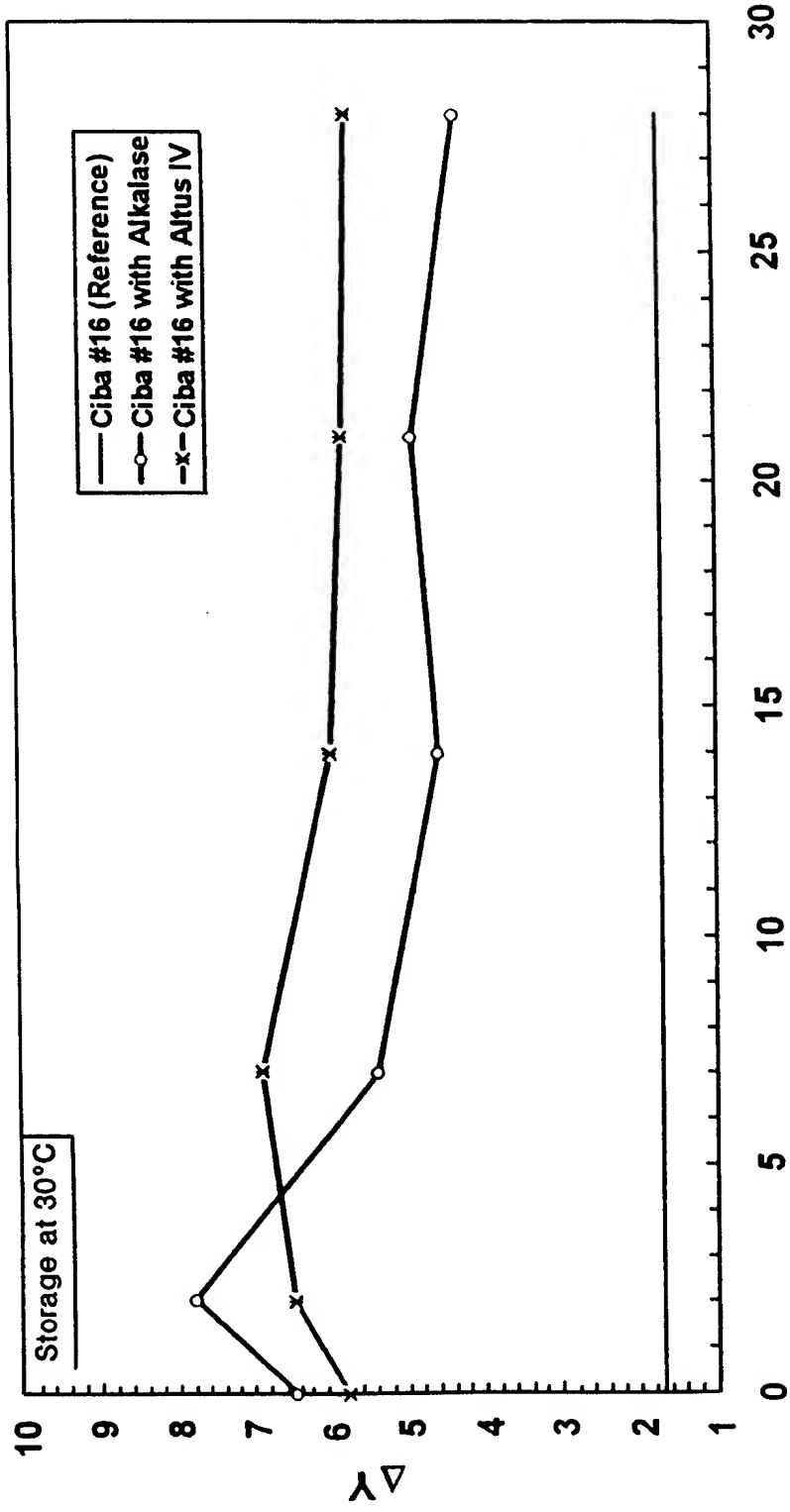


FIG. 3

Δ Y = Lightness after washing – Lightness before washing

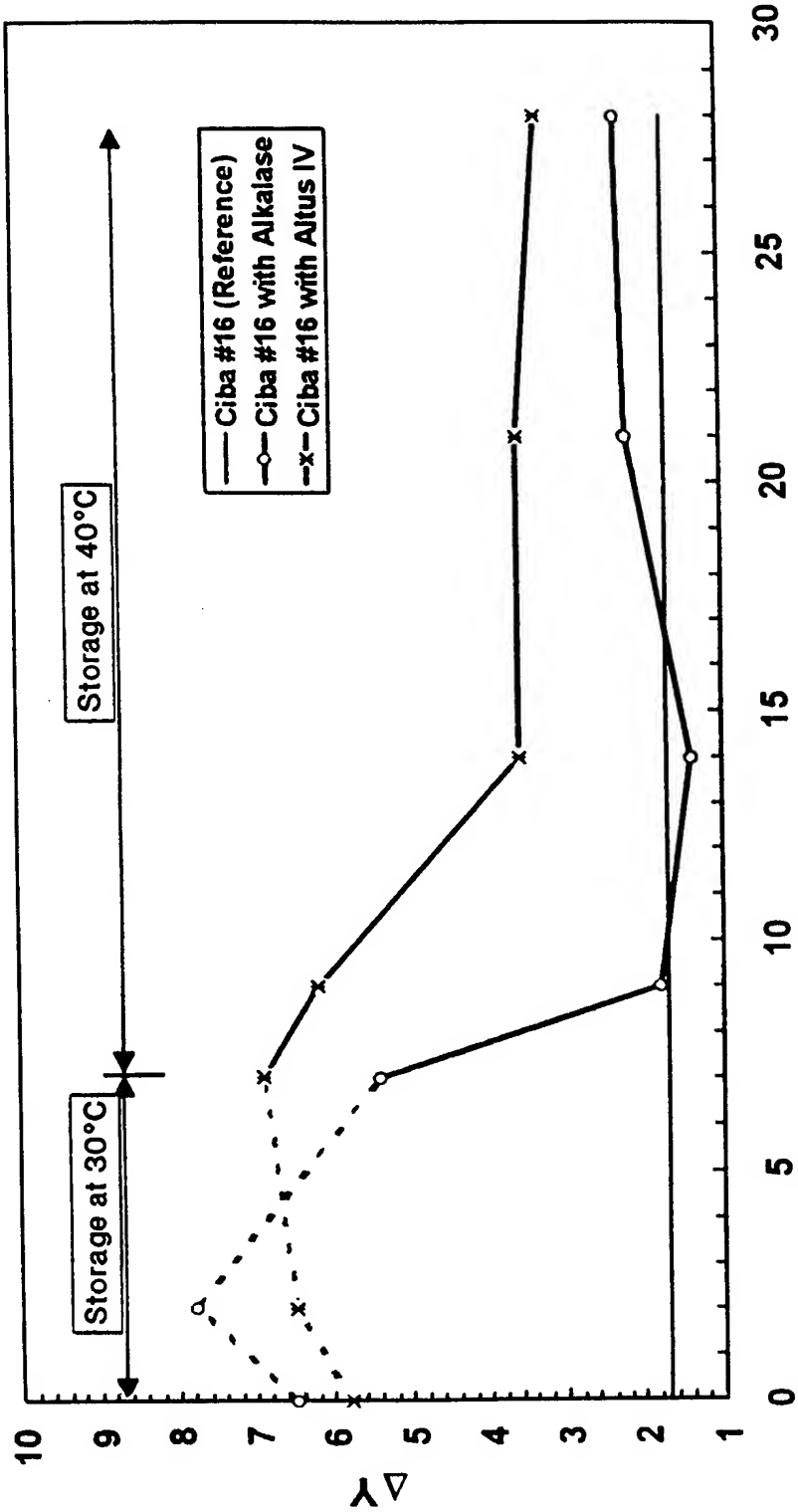


FIG. 4

Δ Y = Lightness after washing – Lightness before washing

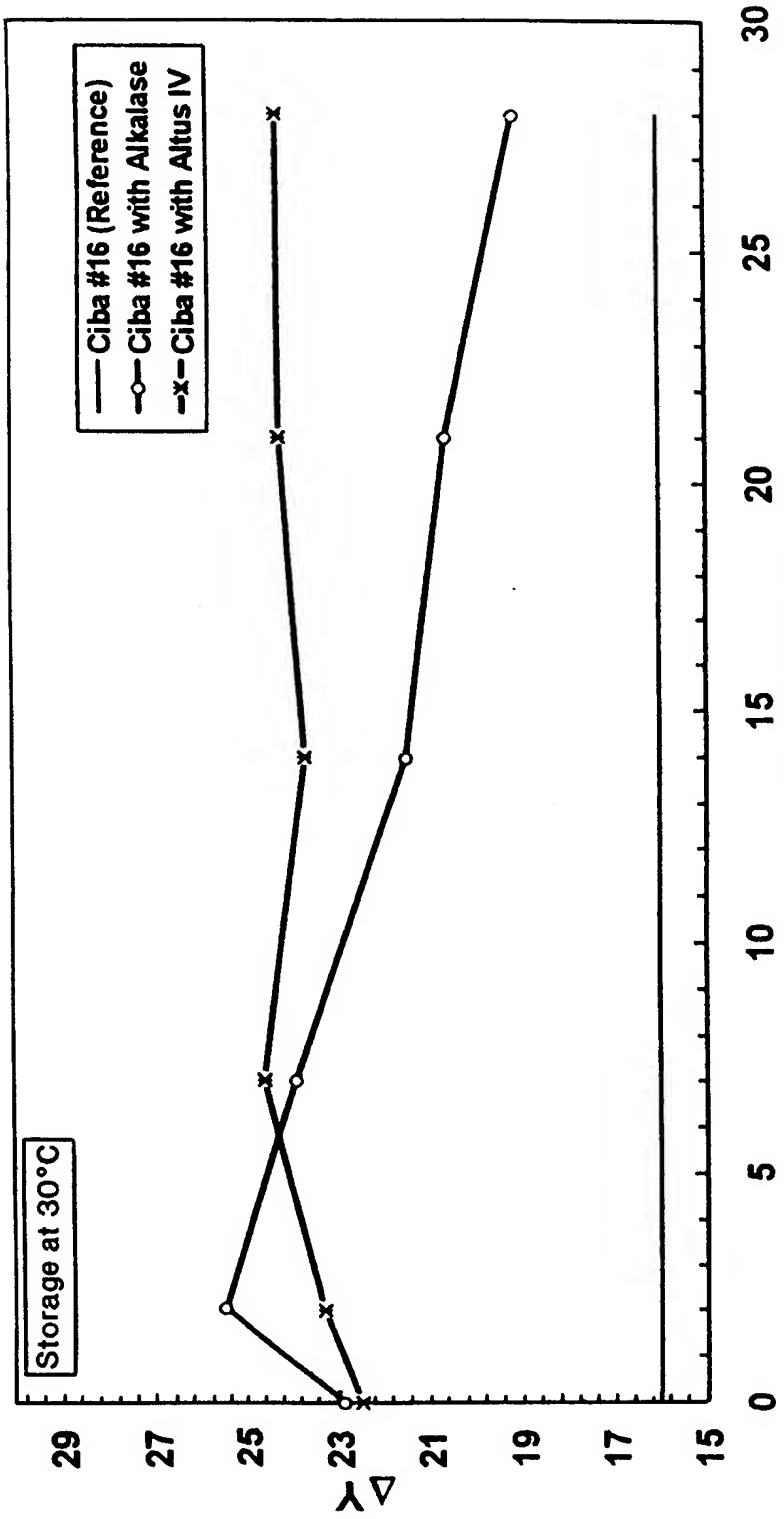

Ciba #16 (Reference)
Ciba #16 with Alkalase
Ciba #16 with Altus IV
Storage at 30°C
ΔY
29
27
25
23
21
19
17
15
0
5
10
15
20
25
30
Storage time (days)
Δ Y = Lightness after washing – Lightness before washing

FIG. 5

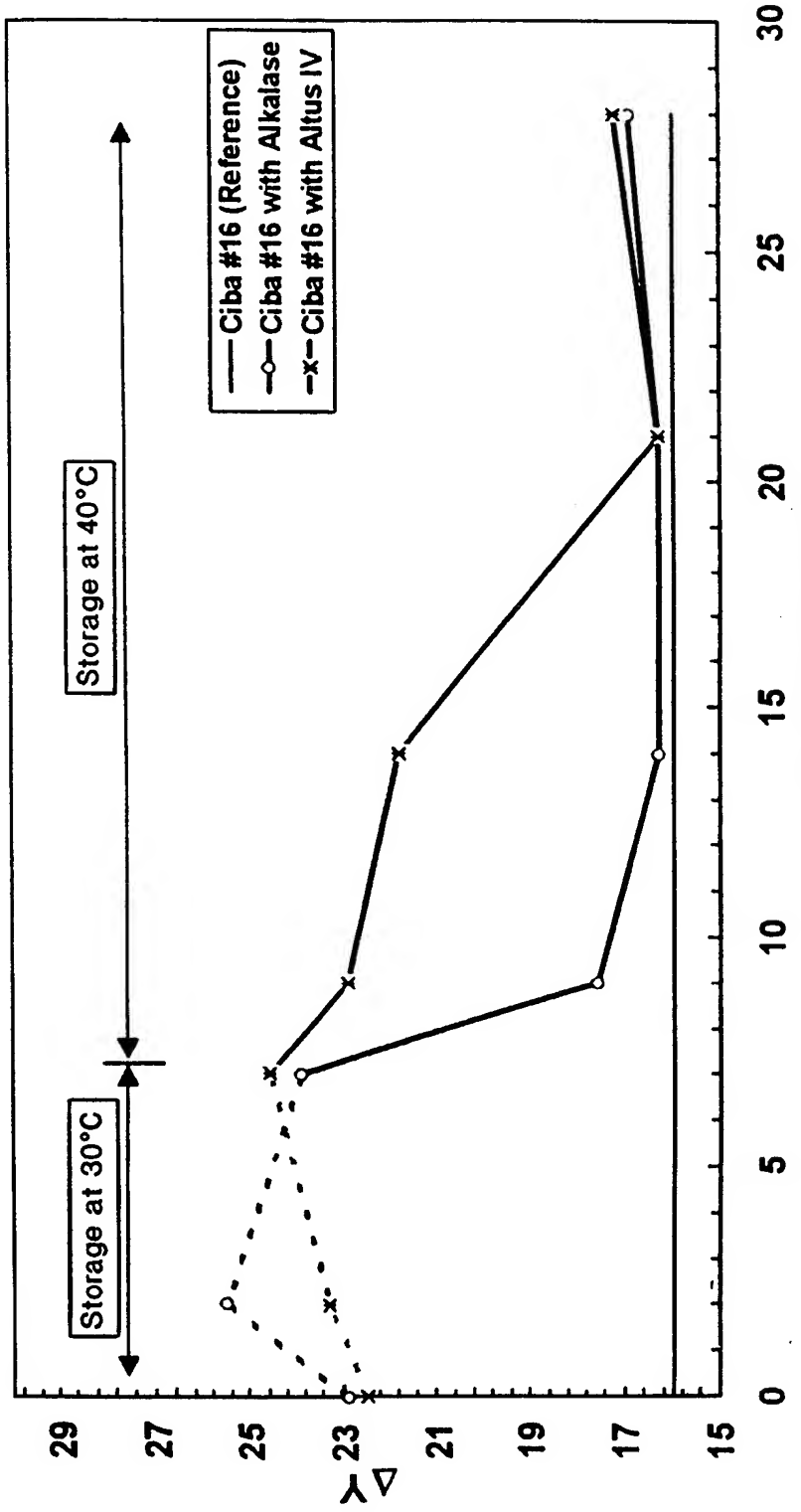


FIG. 6

Δ Y = Lightness after washing – Lightness before washing

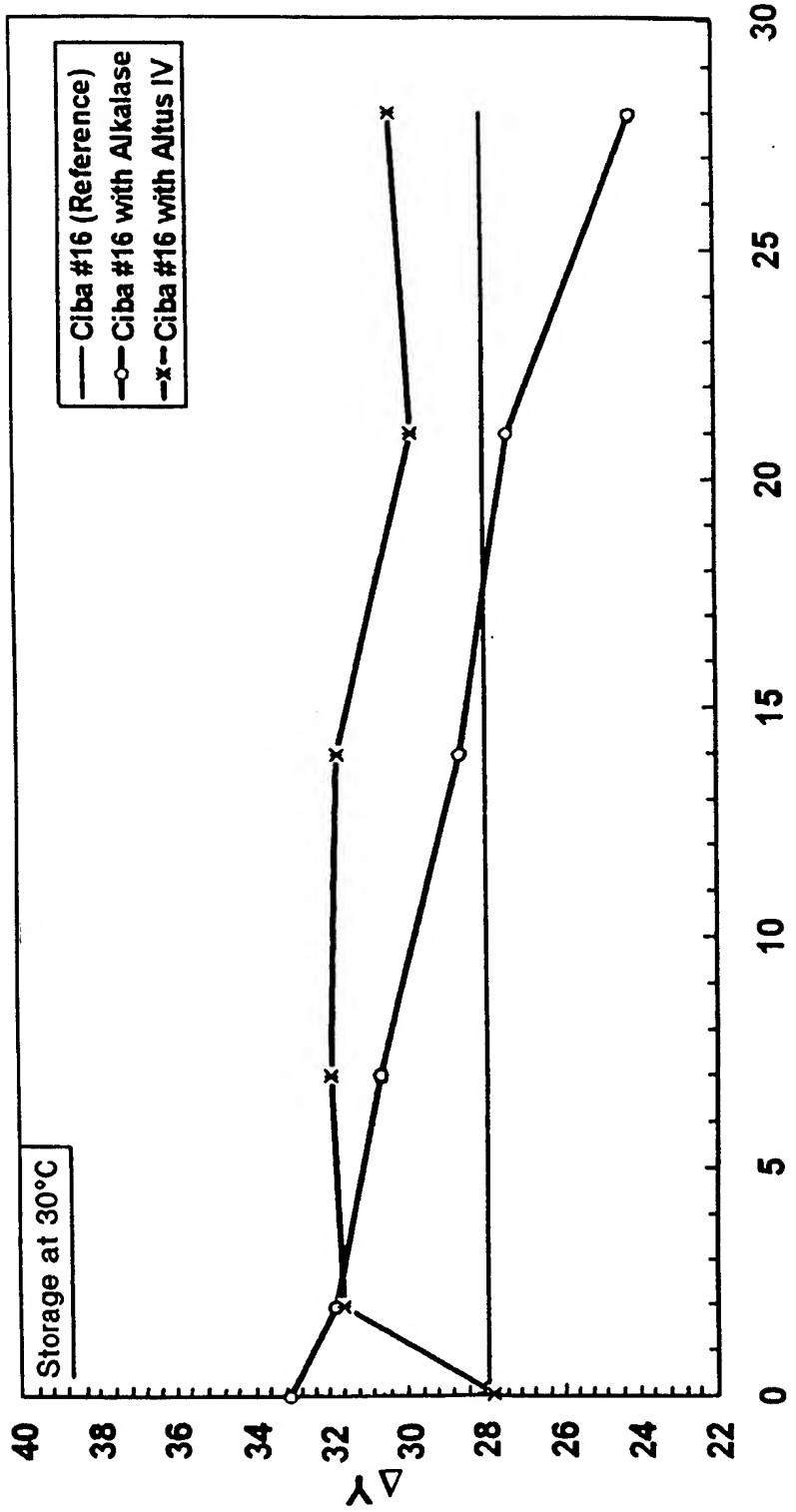


FIG. 7

Δ Y = Lightness after washing – Lightness before washing

FIG. 8

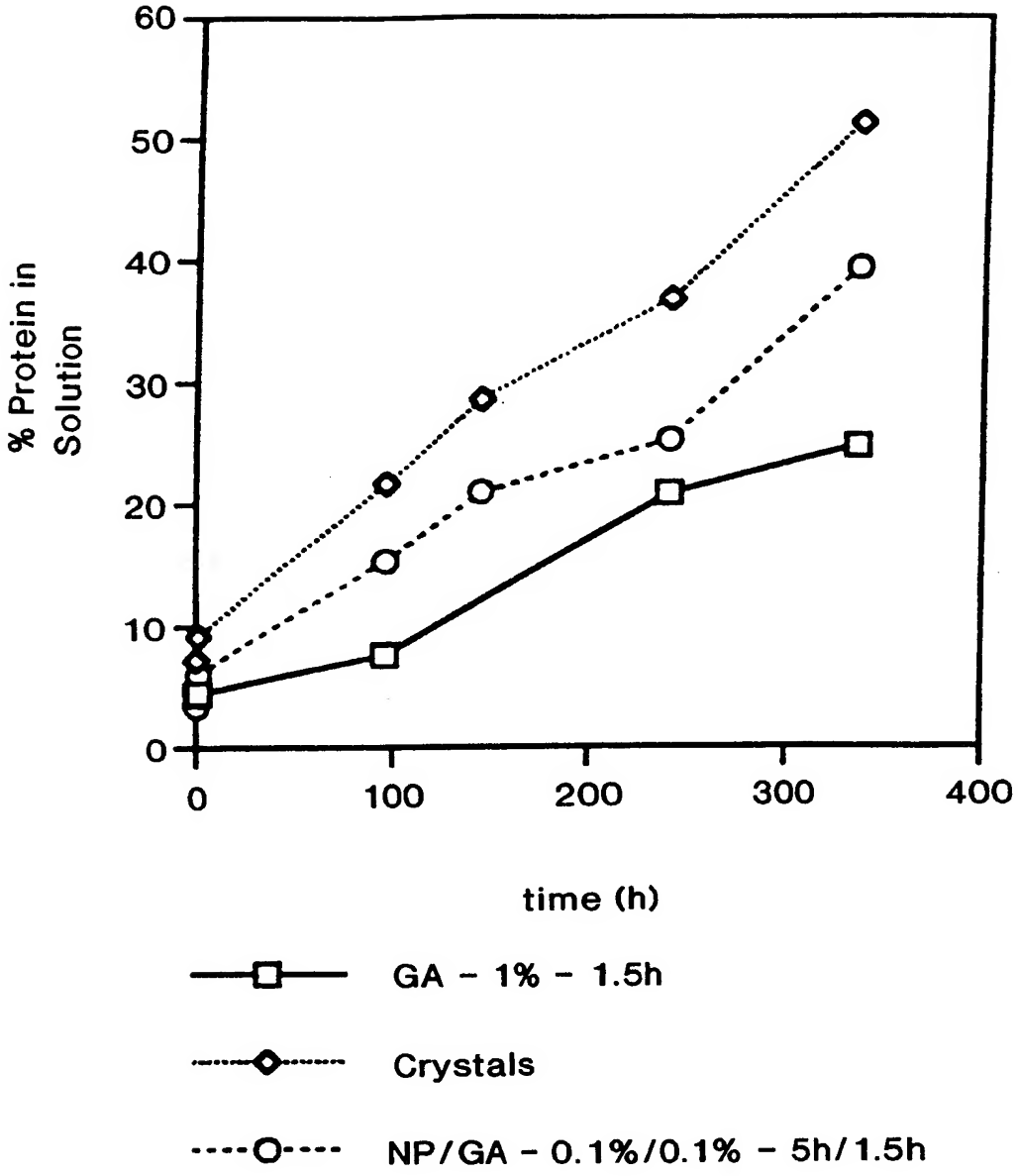
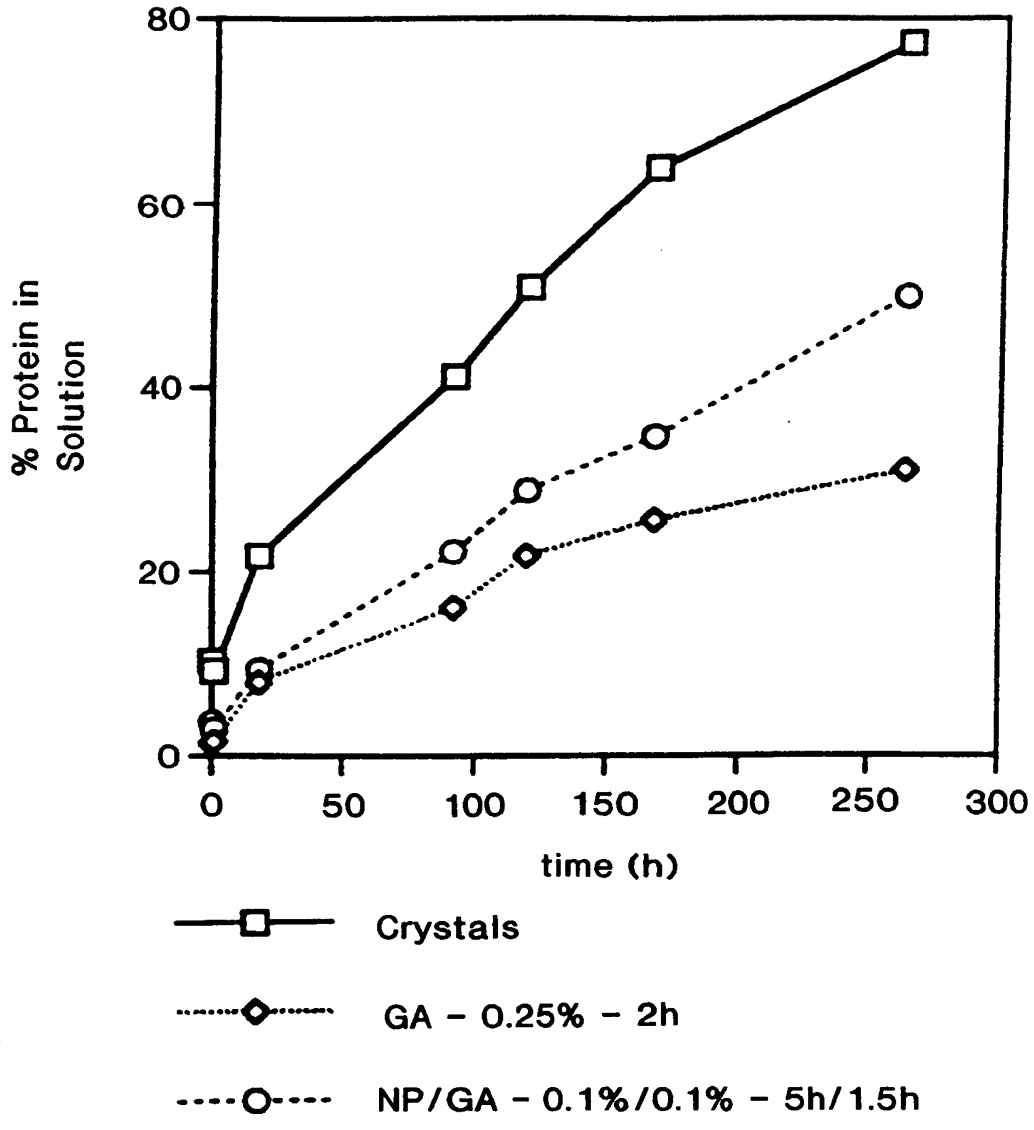

60
50
40
30
20
10
0
% Protein in Solution
0
100
200
300
400
time (h)
GA - 1% - 1.5h
Crystals
NP/GA - 0.1%/0.1% - 5h/1.5h

FIG. 9


80
60
40
20
0
% Protein in Solution
0
50
100
150
200
250
300
time (h)
Crystals
GA - 0.25% - 2h
NP/GA - 0.1%/0.1% - 5h/1.5h

INTERNATIONAL SEARCH REPORT

national Application No
PCT/US 98/07287

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N11/00 C12N9/20 A61K7/00 A61K9/52 C11D17/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K C11D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ALEXEY L. MARGOLIN: "Novel crystalline catalysts" TRENDS IN BIOTECHNOLOGY, vol. 14, no. 7, July 1996, pages 223-230, XP004035759 see page 223, left-hand column, paragraph 2 - page 229, right-hand column, paragraph 1	17, 19-24, 29, 39-57, 65,67, 70, 73-75, 88,92-96
Y	see page 229, right-hand column, paragraph 2 --- -/--	1,12,13, 18, 35-38, 58-66

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 July 1998	12/08/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Montero Lopez, B

1

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INTERNATIONAL SEARCH REPORT

national Application No
PCT/US 98/07287

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	XU, KUI ET AL: "pH control of the catalytic activity of cross - linked enzyme crystals in organic solvents" J. AM. CHEM. SOC. (1996), 118(41), 9815-9819 CODEN: JACSAT;ISSN: 0002-7863, XP002072836 see page 9817, left-hand column, paragraph 3 - page 9818, right-hand column, paragraph 2 see page 9815, right-hand column, paragraph 2 - page 9816, left-hand column, paragraph 2	1,12,13, 18, 35-38, 58-66
X	JIM J. LALONDE ET AL.: "Cross-linked crystals of Candida rugosa lipase: highly efficient catalysts for the resolution of chiral esters" JOURNAL OF THE AMERICAN CHEMICAL SOCIETY., vol. 117, 5 July 1995, pages 6845-6852, XP002041053 DC US see abstract see page 6846, left-hand column, paragraph 3 - right-hand column, paragraph 1 see page 6848, left-hand column, paragraph 2 - page 6850, right-hand column, paragraph 2	17, 19-24, 29, 39-42, 45-48, 57,67, 70, 73-75, 88,92-95
A	WO 92 02617 A (VERTEX PHARMACEUTICALS INCORPORATED) 20 February 1992 see the whole document	
T	WO 98 13119 A (ALTUS BIOLOGICS INC.) 2 April 1998 see page 22, line 9 - line 21 see page 24, line 11 - page 32, line 12	

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

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PCT/US 98/07287

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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WO 9813119 A	02-04-1998	AU 4738197 A	17-04-1998